STUDIES ON THE FORMATION OF MUREIN-BOUND LIPOPROTEIN IN *ESCHERICHIA COLI*

1992

ZHANG

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ABSTRACT

Title of Dissertation: Studies on the formation of murein-bound lipoprotein in Escherichia coli

Wei-Yang Zhang, Candidate, Doctor of Philosophy, 1992.

Thesis directed by: Henry C. Wu, M.D., Ph.D., Professor, Department of Microbiology.

The formation of the covalent linkage between the major outer membrane (Braun's) lipoprotein and the peptidoglycan of Escherichia coli was studied by the isolation and characterization of mutants with reduced bound-form lipoprotein.

Following transposon or nitrosoguanidine mutagenesis, two hundred Escherichia coli mutants with the phenotype of increased sensitivity to sodium dodecyl sulfate and leakiness of periplasmic RNase were each assayed for the amount of murein-bound lipoprotein in each mutant. Five mutants were found to contain no or reduced amounts of murein-bound lipoprotein. Two mutations were shown to be Tn10 and Tn5 insertions in the lipoprotein structural gene (lpp). The lky mutation in strain K86 was shown to be an amber allele of tolA, and the lky mutation in strain K3 was shown to be a new tolB allele. However, the deficiency in the bound-form lipoprotein in strains K3 and K86 did not result from the tol mutations; instead strains K3 and K86 appeared to contain a second mutation near 17 min of the E. coli genome which affected the attachment of lipoprotein to the peptidoglycan. The remaining lky mutant, strain MTA, was shown to be distinct from the colicin-

tolerant mutants at the tolQRAB loci or the leaky mutant at the excC locus. The lky mutation in strain MTA was mapped at the 86 minute on the E. coli chromosome, and a second mutation related to the reduced bound-form lipoprotein was located near 17 minute region of the E. coli chromosome.

A major portion of this thesis is concerned with the characterization of new lpp alleles, constructed by site-specific mutagenesis, which were affected in the formation of bound-form lipoprotein. The relationship between the modification/processing of prolipoprotein and the formation of bound-form lipoprotein was studied in E. coli strains containing lpp alleles altered in the signal sequence of prolipoprotein as well as in an E. coli strain producing an OmpF-Lpp hybrid protein. The unmodified prolipoproteins in mutants lppL20, lppV20, and lppG21, the lipid-modified prolipoproteins in mutant lppT20 and in globomycin-treated wild-type cells, and the lipid-deficient OmpF-Lpp hybrid protein were all found to be covalently attached to the peptidoglycan. These results indicate that neither the lipid modification nor the processing of prolipoprotein is essential for the formation of murein-bound lipoprotein in E. coli.

In contrast, introduction of a charged amino acid residue like Asp or Arg at the 14th position of prolipoprotein not only affected the lipid-modification and processing of the mutant prolipoprotein, but also the formation of murein-bound lipoprotein. Replacement of the Gly14 residue of prolipoprotein with Glu or Lys partially affected the lipid-modification and processing of prolipoprotein;

the peptidoglycan of the lppE14 and lppK14 mutants contained reduced amount of mature lipoprotein but no mutant prolipoprotein. Two lpp mutants, A20I23I24 and A20I23K24, with a reduction in the β-turn secondary structure at the processing site, were defective in both lipid-modification/processing of prolipoprotein and the formation of murein-bound lipoprotein. These results indicate that the formation of murein-bound prolipoprotein requires an absence of a charged residue in the hydrophobic region of the signal sequence and an appropriate secondary structure at the processing site of the signal sequence.

Additional lpp mutations were constructed with specific alterations in the COOH-terminal region of the lipoprotein. As might be expected, substitution of Lys78 with Arg78 prevented the formation of murein-bound lipoprotein. Each of the following single amino acid substitutions in lipoprotein did not significantly affect the formation of bound-form lipoprotein: Asp70 to Glu70, Asp70 to Gly70, Lys75 to Thr75, Tyr76 to His76, Tyr76 to Ile76, and Tyr76 to Leu76. In contrast, mutational alterations of Tyr76 to Cys76, Gly76, Asn76, Pro76, or Ser76 resulted in reduced amounts of the bound-form lipoprotein to levels of 14 to 32% of that in the wild-type strain. A common feature of these lpp COOH-terminal mutations affecting the formation of bound-form lipoprotein was the presence of a β-turn secondary structure instead of a random coil at the COOH-terminal region of all these mutant lipoproteins. Substitution of Tyr76 with Asp or Glu and Arg77 with Asp or Leu also resulted in a reduced formation of

bound-form lipoprotein. These results suggest that the formation of murein-bound lipoprotein requires a positively charged COOH-terminus, and a β-turn secondary structure in the COOH-terminal random coil region interferes with the attachment of the lipoprotein to the peptidoglycan.

A lpp mutant of E. coli with an internal deletion of twenty-one amino acid residues (L37 to A57) of prolipoprotein was identified by sequence analysis following polymerase chain reaction. The prolipoprotein from this lpp internal deletion mutant was lipid-modified and processed, and both the mutant prolipoprotein and its mature free-form lipoprotein containing 57 and 37 amino acid residues, respectively, could form covalent linkage to the peptidoglycan. These results indicate that the internal deletion of the lipoprotein does not affect the formation of murein-bound lipoprotein.

STUDIES ON THE FORMATION OF MUREIN-BOUND LIPOPROTEIN IN *ESCHERICHIA COLI*

by

Wei-Yang Zhang

Dissertation submitted to the Faculty of the Department of Microbiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
1992

DEDICATION

I dedicate this dissertation to my mother, Zhi-Lang Hu (November 10, 1911-December 9, 1964), who gave me life, faith and example.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

I. The cell envelope structure of gram negative bacteria.

The cell envelope of gram-negative bacteria is a highly complex, multilayered structure. The cytoplasmic membrane (also called the inner membrane) is surrounded by a rigid, shape-determining layer of murein (peptidoglycan) to which is anchored the outer membrane. The compartment between the inner and outer membranes, the periplasmic space, contains many hydrolytic enzymes such as RNase I, acid phosphatase, ADP-glucose hydrolase and alkaline phosphatase (Beacham, 1979) and binding proteins that function in the transport of sugars and amino acids such as maltose, histidine and glutamine (Landick and Oxender, 1982). The cytoplasmic membrane, a typical unit membrane composed of phospholipids and proteins, contains the enzymes and the carrier molecules that function in energy production (Ingledew and Poole, 1984), the enzymes for lipid and lipopolysaccharide biosynthesis (Bell et al., 1971; Osborn et al., 1972), and specific transport systems (Saier, 1977).

The nascent glycan chains of the murein sacculus of Escherichia coli consists of alternating N-acetylglucosamine and N-acetylmuramic acid which are linked by β-1,4-glycosidic bonds. Attached to the carboxyl group of each muramic acid by an amide linkage is the peptide, L-alanyl-D-isoglutamyl-meso-diaminopimelyl-D-alanyl-D-alanine. About half of these muropeptides are involved in the cross-linkage of the glycan chains (Schleifer and Kandler,

1972). The peptidoglycan appears to play an essential role in maintaining the cell shape in bacteria (Weidel and Pelzer, 1964).

The outer membrane contains phospholipids, lipopolysaccharide, enterobacterial common antigen, and a number of major protein species including the major outer membrane lipoprotein (Braun and Rehn, 1969), porins (OmpF, OmpC and PhoE), and proteins involved in specific diffusion processes (Lugtenberg and Alphen, 1983). The outer membrane of E. coli serves as a permeability barrier against the entrance of various agents such as hydrophobic antibiotics and dyes into the cell, and prevents the leakage of periplasmic proteins and enzymes (Leive, 1974). Small hydrophilic molecules enter the cell by a diffusion process through the porin channels of the outer membrane (Bavoil et al., 1977) or by specific diffusion processes mediated by outer membrane proteins such as LamB or Tsx which also function as phage receptors (Konisky, 1979).

II. Braun's lipoprotein.

Braun's lipoprotein is a major outer membrane protein of gram-negative bacteria, which was first discovered in its murein-bound form (Braun and Rehn, 1969). In E. coli there are approximately 7.5×10^5 molecules of lipoprotein per cell (DeMartini et al., 1976), one third of which forms covalent linkage with the murein layer and is called murein-bound lipoprotein, and the other two thirds of lipoprotein are called the free-form lipoprotein (Inouye et al., 1972).

While Braun's lipoprotein of E. coli is the most extensively

studied lipoprotein, it is not the only membrane protein with covalently attached lipid. A group of membrane proteins with covalently attached lipid at their amino-termini has been discovered in E. coli and in many other bacterial species in recent years (Hayashi and Wu, 1990). The term "lipoprotein" in this thesis refers to Braun's lipoprotein.

A. Murein-bound lipoprotein.

Braun's lipoprotein is one of the most thoroughly studied membrane proteins. The primary structure of the murein-bound lipoprotein has been determined as shown in Fig. 1 (Braun and Bosch, 1972; Hantke and Braun, 1973). The murein lipoprotein contains 58 amino acids. A unique feature of Braun's lipoprotein and all other lipoproteins in different bacterial species is the presence of the covalently attached glyceride and amide-linked fatty acid at the NH_2-termini of the lipoproteins. There are three fatty acid residues per mole of lipoprotein; two fatty acids are ester-linked to the glycerol residue of the N-terminal glycerylcysteine, while the third fatty acid is amide-linked to the α-amino group of the N-terminal diglyceride-cysteine. The composition of the ester-linked fatty acids corresponds to that of the cellular phospholipids, while the amide-linked fatty acid is mainly palmitic acid. The murein-bound lipoprotein forms a covalent linkage with peptidoglycan through the ϵ-NH_2 group of its COOH-terminal lysine residue. Edman degradation of the murein-peptide (GlcNac-MurNac-L-Ala-D-Glu-DAP-Lys-Arg), obtained by the

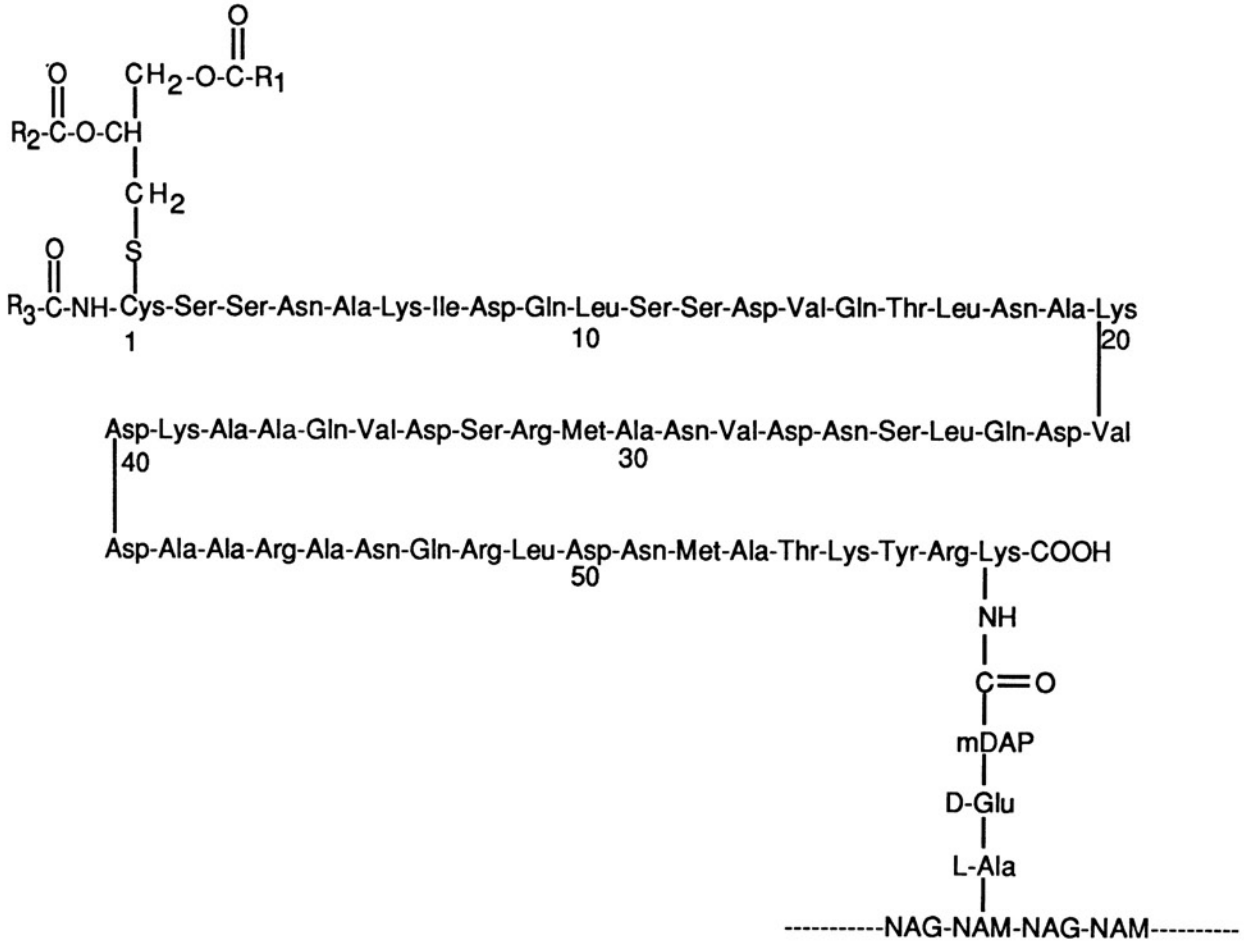


Fig. 1. The primary structure of the murein-bound lipoprotein of E. coli (Braun and Bosch, 1972). mDAP, meso-diaminopimelic acid; NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine. R_1, R_2, and R_3 represent hydrocarbon chains of fatty acids.

enzymatic degradation of the murein-lipoprotein complex with pronase and lysozyme, indicates that the ϵ-amino group of the C-terminal lysine residue of lipoprotein is linked to the carboxyl group of the optically active L-center of the meso-diaminopimelic acid of murein (Braun and Wolf, 1970).

B. Free-form lipoprotein.

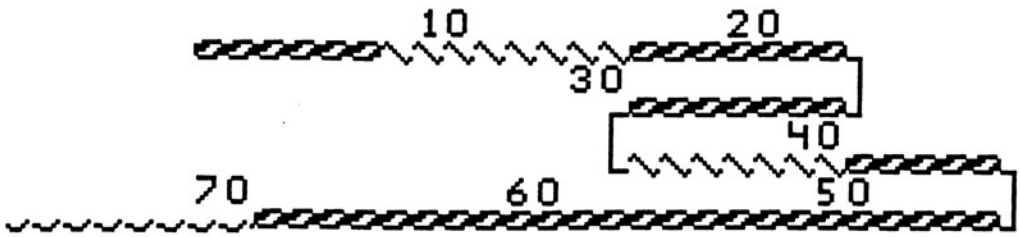
The free-form lipoprotein, which was discovered by Inouye and his co-workers (Inouye et al., 1972), has the same chemical structure as that of the murein-bound lipoprotein except that the COOH-terminal lysine residue of the free-form lipoprotein is not covalently linked to the peptidoglycan. The purified lipoprotein was found to have a high α-helix content (Inouye et al., 1976). The secondary structure of the prolipoprotein as predicted by the Chou-Fasman rules (Chou and Fasman, 1978) is shown in Fig. 2.

The lipoprotein appears to have hydrophobic interactions with the outer membrane through their N-terminal three fatty acid residues. In addition, it has both covalent and noncovalent interactions with the peptidoglycan through the C-terminal lysine residue (Choi et al., 1987). Thus Braun's lipoprotein may play an important role in the maintenance of the structural integrity of the cell envelope of the gram negative bacteria.

C. Biogenesis of lipoprotein in E. coli.

1. Modification of prolipoprotein.

The murein lipoprotein is first synthesized as a precursor,



= Helix = Sheet = Turn = Coil

Fig. 2. The secondary structure of E. coli prolipoprotein as predicted by the Chou-Fasman rules (Chou and Fasman, 1978).

the prolipoprotein, which contains a 20 amino acid residue signal peptide (Inouye et al., 1977). The prolipoprotein undergoes a series of modification and processing reactions to become the mature free-form lipoprotein and assembles into the outer membrane.

The discovery of globomycin, a specific inhibitor of the prolipoprotein signal peptidase or signal peptidase II (Inukai et al, 1978), represents a major breakthrough in the biochemical studies of lipoprotein biogenesis. The accumulation of glyceride-modified prolipoprotein in globomycin-treated wild-type E. coli cells provides the evidence that lipid modification of prolipoprotein precedes the processing reaction (Hussain et al., 1980). Processing of prolipoprotein by the globomycin-sensitive signal peptidase II requires prior modification of prolipoprotein to form glyceride-prolipoprotein (Tokunaga et al., 1982b). A pathway for the biosynthesis of lipoprotein in E. coli was postulated by Tokunaga et al. (Tokunaga et al., 1982b, Fig. 3).

The formation of the glyceride-cysteine in lipoprotein is a multi-step process. The first step is the transfer of a glyceryl moiety from phosphatidylglycerol (PG) to unmodified prolipoprotein by an enzyme called prolipoprotein glyceryl transferase; the carbon 1 of the nonacylated sn-glycerol in PG forms a thioether linkage with the sulfhydryl group of the cysteine residue in prolipoprotein (Chattopadhyay and Wu, 1977).

The second step of the lipid-modification of prolipoprotein is the O-acylation of the glyceryl-prolipoprotein by the glyceryl-prolipoprotein O-acyl transferase(s). The fatty acids in

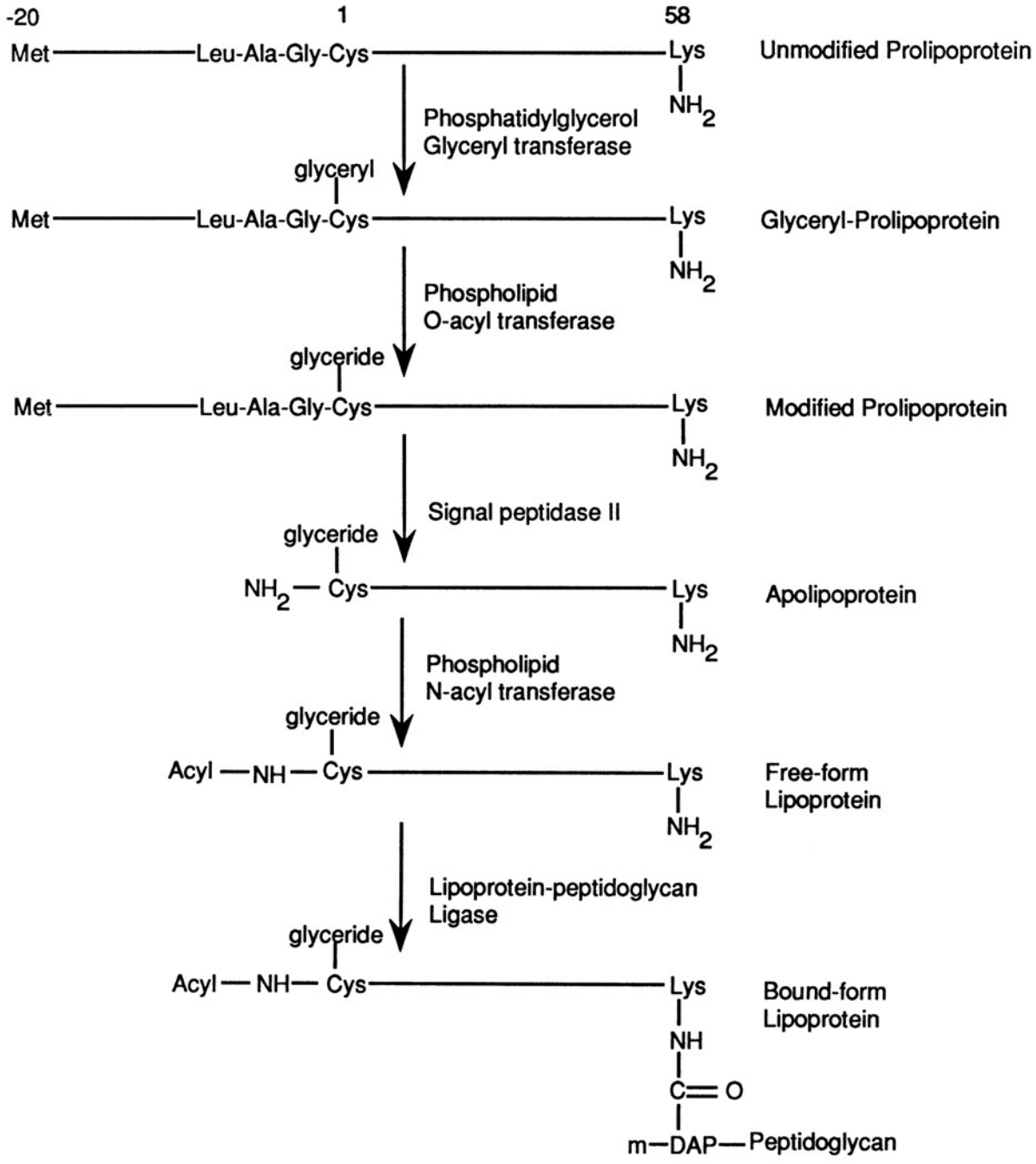


Fig. 3. Biosynthetic pathway of E. coli lipoprotein. m-DAP, meso-diaminopimelic acid.

lipoprotein are derived from pre-existing fatty acids in the phospholipids and are not directly derived from acyl-CoA or acyl-ACP (Lai et al., 1980). Studies of the incorporation of acyl moieties of phospholipids into the lipoprotein in intact cells of E. coli by phospholipid vesicle fusion indicate that the major glycerophosphatides [PG, phosphatidylethanolamine (PE), or cardiolipin (CL)] are equally efficient as the acyl donor for the O-acylation of glyceryl-prolipoprotein (Lai and Wu, 1980).

2. Processing of prolipoprotein.

Processing of glyceride-modified prolipoprotein is catalyzed by a unique enzyme, signal peptidase II, which requires a glyceride-cysteine at the cleavage site (Tokunaga et. al., 1982a). Prior modifications of the prolipoprotein with glyceride is a prerequisite for the processing by the signal peptidase II. Substitution of Cys21 with Gly not only eliminates the lipid modification but also the processing of prolipoprotein (Inouye et al., 1983). Mutant prolipoproteins LppD14 (Lin et al., 1978), R14 (Gennity et al., 1990), L20, V20 (Pollitt et al., 1986), G21 (Inouye et al., 1983) and A20I23I24 (Inouye et al., 1986) are neither modified with lipid nor processed by signal peptidase II. In the region of the modification/cleavage site of prolipoprotein signal peptides, the sequence of Leu-Ala/Ser-Gly/Ala-Cys has been shown to be highly conserved among various bacterial lipoprotein precursors (Hayashi and Wu, 1990). This consensus sequence is also referred to as the lipoprotein box. The gene for the signal

peptidase II (lsp) was cloned and sequenced (Tokunaga et al., 1983; Innis et al., 1984). The lsp gene, located at 0.5 min of the E. coli chromosome (Regue et al., 1984), is part of an operon, x-ileS-lsp-orf149-orf316 (Miller et al., 1987). Processing of lipid-modified prolipoprotein is uniquely sensitive to inhibition by globomycin (Inukai et al., 1978). It is known that the structure of the prolipoprotein affects the degree of inhibition of processing by globomycin. Thus, processing of lipid-modified prepenicillinase is inhibited to a lesser extent than that of prolipoprotein by the same concentration of globomycin (Hayashi and Wu, 1983). Likewise, a mutation near the COOH terminus of lipoprotein (Arg68 to Cys68) renders the processing of mutant prolipoprotein more resistant to inhibition by globomycin (Giam et al, 1984b).

3. N-acylation of apolipoprotein.

The processing of glyceride-prolipoprotein results in the formation of apolipoprotein which undergoes N-acylation to become the mature free-form lipoprotein. The enzyme that catalyzes this reaction is called phospholipid apolipoprotein transacylase or N-acyl transferase which is located in the inner membrane (Gupta and Wu, 1991). An in vitro assay of the N-acyl transferase was developed by Gupta and Wu (Gupta and Wu, 1991) based on the finding that the signal peptidase is thermostable whereas N-acyl transferase is not (Hussain et al, 1982). It has been postulated that the N-acyl fatty acid attached to the amino terminus of

lipoprotein is derived from the fatty acid at the 1-position of PE (Jackowski and Rock, 1986). A mutant strain of E. coli (pss::Tn5) containing a null mutation in the phosphatidylserine synthase gene (pss) was shown to form mature lipoprotein containing amide-linked fatty acid. In vitro assay of apolipoprotein N-acyltransferase using membranes either from the mutant or the wild-type strain as the source of both the enzyme and the acyl donor revealed that both membranes were equally active in the conversion of apolipoprotein to lipoprotein, even though the mutant cell membrane contained negligible amounts of PE. Thus PE is not the sole acyl donor for the N-acylation of apolipoprotein (Gupta et al., 1991).

4. Translocation of lipoprotein.

A functional secretory machinery of E. coli, which includes the SecA protein (Oliver and Beckwith, 1981), the SecB protein (Kumamoto and Beckwith, 1983), the SecD protein (Gardel et al., 1987), the SecE protein (Schatz et al., 1989), the SecF protein (Gardel et al., 1990) and the SecY protein (Ito et al., 1983), has been shown to be essential for protein secretion in general. The export of lipoprotein in E. coli has been demonstrated to require functional SecA, SecY, SecD, SecE, and SecF proteins (Hayashi and Wu, 1985; Sugai and Wu, 1992). However, the export of murein lipoprotein occurs normally in an E. coli secB::Tn5 mutant (Watanabe et al., 1988). Thus the murein lipoprotein belongs to the so-called SecB-independent group of exported proteins which include ribose-binding protein and alkaline phosphatase (Kumamoto

and Beckwith, 1985). The exported prolipoprotein undergoes extensive modification and processing prior to its assembly to the outer membrane. On the basis of these findings a common export pathway for lipoprotein and nonlipoprotein precursors in E. coli has been postulated (Sugai and Wu, 1992, Fig. 4). However, neither the modification nor the processing of prolipoprotein is required for the translocation of prolipoprotein from the cytoplasmic membrane to the outer membrane. The unmodified prolipoprotein of mutant lppD14 is translocated to the outer membrane (Lin et al., 1980a). Likewise, the modified prolipoprotein in globomycin-treated wild-type E. coli cells is also translocated to the outer membrane (Inukai and Inouye, 1983) and covalently linked to the peptidoglycan (Inukai et al., 1979; Ichihara et al., 1982).

5. Conversion of free-form lipoprotein to murein-bound lipoprotein.

(1). Attachment of lipoprotein to the peptidoglycan.

When E. coli cells are pulse-labeled with [^{14}C]arginine for 4 min, most of the [^{14}C]arginine-labeled lipoprotein in the cell envelope is present as the free form. After a chase with non-radioactive arginine for ten minutes, a portion of the free-form lipoprotein is converted into the bound form. The ratio of the radioactivity in the free form to that in the bound form reaches a constant level (1:0.4) after chase for one doubling time. These results indicate that the free-form lipoprotein is the precursor of the murein-bound form, and there appears to be an equilibrium between these two forms of lipoprotein (Inouye et al., 1972). The

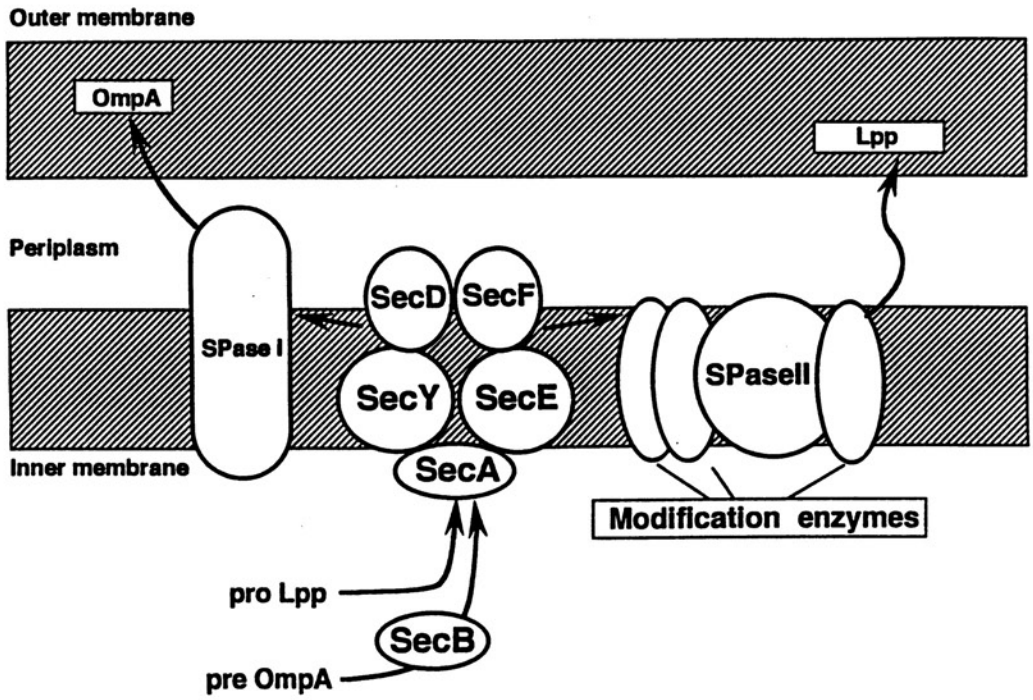


Fig. 4. A common export pathway for lipoprotein and nonlipoprotein precursors in E. coli (Sugai and Wu, 1992). Export of prolipoprotein requires functional SecA, SecY, SecE, SecD, and SecF proteins, but does not require a functional SecB protein. The divergence of the export pathway for lipoproteins and nonlipoproteins occurs after the preproteins have encountered functional SecD/SecF proteins.

conversion of free- to bound-form lipoprotein has been shown not to be affected by an inhibition of protein synthesis with amino acid starvation or chloramphenicol (Inouye et al., 1972). Furthermore, significant conversion of the free form to the bound form is observed even in the presence of carbonyl cyanide m-chlorophenylhydrazone, an energy uncoupler (Inouye et al., 1972). The content of lipoprotein is increased in a strain merodiploid for the lpp gene. In this strain, the free-form lipoprotein is doubled in the outer membrane as compared to the haploid strain (Movva et al., 1978). However, the amount of the bound-form lipoprotein is the same in both strains, suggesting that the attachment sites of the peptidoglycan for the bound-form lipoprotein are saturated in a haploid strain.

(2). Lipoprotein-peptidoglycan ligase.

Braun suggested that the penicillin-insensitive carboxypeptidase II (L,D-carboxypeptidase) might be the lipoprotein-peptidoglycan ligase (Braun, 1975). Penicillin-insensitive L,D-carboxypeptidases have been identified in E. coli (Izaki and Strominger, 1968; Metz et al., 1986). These enzymes remove the terminal D-ala from the tetrapeptides of murein and thus create tripeptide acceptor sites, which are used for the attachment of lipoprotein (Ursinus et al., 1992). The conversion of free- to bound-form lipoprotein is not affected by penicillin G (Braun et al., 1974) or penicillin FL 1060 (Braun and Wolff, 1975). Carboxypeptidase II activity is highest in newly divided or about-

to-divide cells (Beck and Park, 1976).

The in vivo function of the carboxypeptidase II and the enzymatic mechanism of the conversion of the free-form lipoprotein to the murein-bound lipoprotein are unclear. The attachment of free-form lipoprotein to the peptidoglycan may be a two-step process. The first step is the removal of the terminal D-ala from the tetrapeptides of murein to generate the tripeptide acceptor sites for the attachment of lipoprotein, followed by the linkage of the free-form lipoprotein to the tripeptide acceptors. Either two distinct enzymes (a L,D-carboxypeptidase and a peptidoglycan-lipoprotein ligase) or a single enzyme with two activities (carboxypeptidase and ligase) may be involved in this process. Alternatively, the conversion of the free-form lipoprotein to the murein-bound lipoprotein might be a L,D-transpeptidation. As a L,D-transpeptidase, the lipoprotein-peptidoglycan ligase catalyzes the cleavage of the peptide bond between the L-optical center of m-DAP and D-ala of the tetrapeptides with the concomitant formation of a peptide bond between the COOH group of the L-center of m-DAP and the ϵ-amino group of the carboxyl-terminal lysine residue of the lipoprotein.

(3). Inhibition of the formation of bound-form lipoprotein by certain D-amino acids and β-lactam antibiotics.

Lark and Lark reported that certain D-amino acids, such as D-methionine and D-cysteine, caused spheroplast formation of Alcaligenes cells, and these amino acids exerted a synergistic

effect with penicillin (Lark and Lark, 1959). Tsuruoka et al. showed that D-amino acids were incorporated into the peptidoglycan of E. coli cells under conditions in which both protein and cell wall synthesis were inhibited (Tsuruoka et al., 1984). When D-^{14}C-amino acid such as D-[^{14}C]methionine or DL-[^{14}C]cysteine were incubated with E. coli cells in phosphate buffer deprived of nutrients, the radioactivity was rapidly incorporated into the cells, and an appreciable part of the radioactivity was recovered in the sodium dodecyl sulfate-insoluble peptidoglycan fraction. Incorporation of D-[^{14}C]methionine into peptidoglycan was not inhibited by the addition of 500 μg/ml of benzylpenicillin or ampicillin, which would inhibit the cross-linking of peptidoglycan. Certain β-lactam compounds that possess a terminal D-amino acid moiety were also incorporated into E. coli peptidoglycan through the D-amino groups of the side chains (Tsuruoka et al., 1985). The formation of the covalent linkage between β-lactam compounds carrying the D-amino acid and the peptidoglycan was insensitive to β-lactam antibiotics such as benzylpenicillin and ampicillin, and therefore not catalyzed by a D,D-transpeptidase involved in the biosynthesis of peptidoglycan. These D-amino acids also inhibited the formation of murein-bound lipoprotein in E. coli (Tsuruoka et al., 1984). Presumably D-amino acids and β-lactam compounds that carry a D-amino acid moiety are covalently linked to the peptidoglycan by an L,D-transpeptidase similar to (or identical with) the enzyme which catalyzes the formation of the murein-bound lipoprotein, and therefore competitively inhibit the attachment of

lipoprotein to the murein.

III. E. coli or Salmonella typhimurium mutants with reduced murein-bound lipoprotein.

A. E. coli lpp mutations affecting the formation of bound-form lipoprotein.

1. Signal-sequence mutations of prolipoprotein.

The first signal sequence mutation that affects the attachment of lipoprotein to the murein is lppD14 (previously known as mlpA, Wu and Lin, 1976; Yem and Wu, 1977). This mutant contains an unmodified prolipoprotein with a single amino acid substitution of glycine by aspartic acid at position 14 of the prolipoprotein (Lin et al., 1978). As a consequence of this single amino acid replacement, post-translational modifications and processing of prolipoprotein are severely affected. The lppD14 mutant exhibits phenotypes of increased sensitivity to EDTA, sodium dodecyl sulfate (SDS) and other detergents, leakiness of periplasmic RNase, and blebbing of the outer membrane upon Mg^{++} starvation (Yem and Wu, 1978). The murein sacculus of mutant lppD14 contains a greatly reduced amount of mature lipoprotein but no prolipoprotein (Lin et al., 1980b). However, the unmodified prolipoprotein of the mutant is translocated to the outer membrane (Lin et al., 1980a). Because of the defect in lipid modification of the mutant prolipoprotein, the lppD14 mutant also shows increased resistance to globomycin owing to its failure to accumulate glyceride-modified prolipoprotein in the cytoplasmic membrane of globomycin-treated

cells (Lai et al., 1981).

2. Deletion of COOH-terminal lysine residue.

The COOH-terminal lysine residue is most likely essential for the formation of murein-bound lipoprotein since it participates in the covalent linkage between the lipoprotein and the peptidoglycan in E. coli, and it is conserved as the COOH-terminal amino acid residue in homologs of Braun's lipoprotein from five different enterobacteriaceae species including E. coli, Proteus mirabilis, Morganella morganii, Erwinia amylovora and Serratia marcescens, and in Pseudomonas aeruginosa lipoprotein I (Fig. 5., Yu, 1987; Duchêne et al., 1989). It has been suggested that Braun's lipoprotein exists as a trimer in the periplasmic space of E. coli, and interacts with the peptidoglycan both covalently and noncovalently through the protein domain and with the outer membrane through the lipid domain at the amino terminus (Yu et al., 1984). Two hybrid genes encoding lipid-deficient OmpF-Lpp hybrid proteins were constructed to study the molecular assembly of the lipoprotein on the peptidoglycan layer (Choi et al., 1987). One gene codes for a hybrid protein in which the glyceride-cysteine residue in lipoprotein is replaced with the NH_2-terminal Ala-Glu residues of the OmpF protein. The other gene codes for a similar lipid-free OmpF-Lpp hybrid protein with a deletion of the COOH-terminal lysine residue. Analysis of the oligomeric structures and the subcellular localization of these two proteins revealed that while the deletion of the COOH-terminal lysine residue did not interfere with the

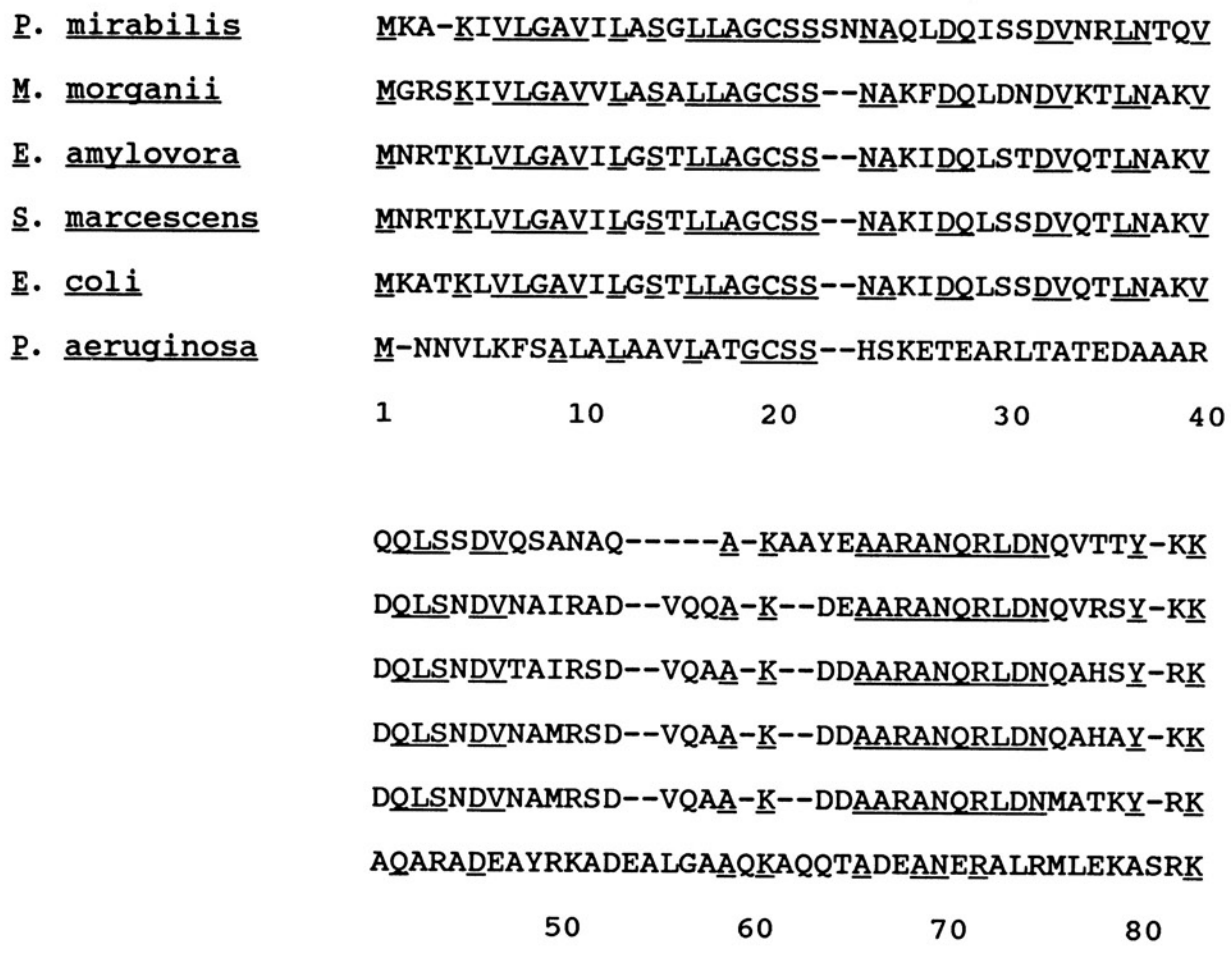


Fig. 5. Amino acid sequences of prolipoproteins from E. coli and four other enterobacteriaceae species (Yu, 1987), and P. aeruginosa (Duchêne et al., 1989). The underlined amino acid residues are conserved amino acid residues among these five enterobacteriaceae species. Amino acid residues in P. aeruginosa prolipoprotein identical to those in the prolipoproteins of enterobacteriaceae are also underlined.

trimerization of the mutant hybrid protein, it did interfere with both the noncovalent and the covalent interaction of the hybrid protein with the murein layer. These results indicate that the COOH-terminal lysine residue is involved in both the covalent and noncovalent interactions of the lipoprotein with the peptidoglycan.

B. S. typhimurium lkyD mutants with reduced amount of bound-form lipoprotein.

1. Phenotypes of lkyD mutants of S. typhimurium.

The lkyD mutants of S. typhimurium were found to excrete significant amounts of periplasmic enzymes and proteins including RNase I, acid hexose phosphatase, ribose binding protein, and histidine binding protein (Weigand and Rothfield, 1976). Other characteristic phenotypes of the lkyD mutants are the increased sensitivity to detergents such as SDS and deoxycholate and to rifampicin, and the formation of outer membrane blebs over the septal and polar regions of the mutant cells. The formation of outer membrane blebs in lkyD mutants reflects a defect of these mutant cells in the invagination of the outer membrane during the formation of the division septum. During the process of septation in lkyD cells, the outer membrane fails to follow the cytoplasmic membrane and murein layers into the developing septum, and bulges outward over the septal region, leading to the formation of large blebs over the septal region of the cell. Complete cell separation leaves a large bleb of the outer membrane attached to the pole of one of the progeny cells (Weigand et al., 1976).

2. Mapping of lkyD mutations.

Two mutants of S. typhimurium have been described which have the LkyD characteristics. They are strains R71 (Weigand and Rothfield, 1976) and Rts34 (Fung et al., 1980). The results of P22 transduction suggested that the R71 lkyD mutation maps close to proB25 (Fung, 1978). Strain Rts34 is temperature-sensitive in lkyD phenotypes. Results obtained in a three-factor cross using the markers lkyD, gpt, and proB suggested that the lkyD mutation in Rts34 is located counterclockwise to both the gpt and proB genes (Fung, 1978). The map position of the lkyD allele of Rts34 and R71 were shown to be approximately 5.5 to 6.0 min and 6.0 to 6.5 min on the S. typhimurium chromosome, respectively (Fung, 1978).

3. Biochemical defect in lkyD mutants.

The defective invagination of the outer membrane during the formation of the division septum in lkyD strains may result from a deficiency in the linkage between the outer membrane and the murein layer. The bound-form lipoprotein could provide such a linkage. The ratio of bound-form to free-form lipoprotein is four-fold lower in lkyD mutants than that of the wild-type stain, while the total lipoprotein (free plus bound form) is approximately the same (Weigand et al., 1976). These results suggest that the morphogenetic abnormality of lkyD mutants results from a defect in the formation of murein-bound lipoprotein.

IV. Lipid-modified membrane proteins in bacteria.

Since the discovery of Braun's lipoprotein in E. coli, many membrane proteins with covalently attached lipid have been identified in E. coli and in other bacteria. These lipid-modified membrane proteins are collectively called as lipoproteins. The structures, functions and biogenesis of many lipoproteins have been investigated in recent years.

A. Structural characterization of lipoproteins.

By labeling cells with [^{3}H]glycerol and [^{3}H]palmitic acid several new species of lipoproteins, in addition to Braun's lipoprotein, were found in the E. coli cell envelope (Ichihara et al., 1981). These new lipoproteins were immunologically distinct from Braun's lipoprotein. Four of these lipoproteins were found to be localized in the outer membrane, while two were found in the cytoplasmic membrane.

Lipoproteins with covalently attached lipid at the amino-terminus were also found in other gram negative bacteria as well as in gram positive bacteria. They include the homologs of Braun's lipoprotein in various gram-negative bacteria, extracellular enzymes (β-lactamases from Bacillus licheniformis, Staphylococcus aureus and Bacillus cereus; pullulanases from Klebsiella pneumoniae and K. aerogenes; chitobiase of Vibrio harveyii; and β-1,4-endoglycanase from Pseudomonas solanacearum), plasmid-encoded TraT proteins required for conjugation, lysis proteins for the release of bacteriocins, and lipoproteins of unknown functions such as Pal (peptidoglycan associated lipoprotein), RplA, RplB, NlpB, and Orf17

in E. coli; and Pal and Pal cross-reacting protein in Haemophilus influenzae (summarized in Hayashi and Wu, 1990).

These lipoproteins having a common structure are synthesized as prolipoproteins. The signal sequences of these prolipoproteins contain a consensus sequence, Leu-Ala(Ser)-Gly(Ala)-Cys at the carboxyl-terminal region of the signal sequences (Hayashi and Wu, 1990). They are predicted to have a β-turn secondary structure immediately following the cleavage site at the +2 or +3 positions (Hayashi and Wu, 1990).

B. Biogenesis of lipoproteins.

While most of the studies on the modification and processing of prolipoprotein were carried out with E. coli Braun's lipoprotein, other lipoproteins in E. coli presumably share this modification and processing system, and similar systems exist in other bacterial species. The precursor forms of the new lipoproteins including Pal accumulated as lipid-modified prolipoproteins in E. coli B cells treated with globomycin (Ichihara et al., 1981). These results indicate that the lipid-modification of these new lipoproteins precedes the processing reaction, and the processing of these new lipoproteins is catalyzed by signal peptidase II. E. coli lpp gene has been expressed in B. subtilis resulting in the formation of mature free-form lipoprotein (Hayashi et al., 1985). Like in E. coli cells, the processing of lipid-modified prepenicillinase and prolipoprotein (Braun's) is inhibited by globomycin in B. subtilis (Hayashi and Wu, 1983).

These results suggest that similar modification and processing enzymes exist in B. subtilis. Using an in vitro assay for the post-translational modification and processing of E. coli prolipoprotein, the activities of prolipoprotein modification enzymes and signal peptidase II have been detected in the detergent solubilized cell envelopes of B. subtilis and Streptomyces (Hayashi and Wu, unpublished data).

C. Functions of lipoproteins.

There are no common functions for such a diverse group of bacterial lipoproteins. Some lipoproteins may play an important role in the maintenance of the cell envelope structure such as Braun's lipoprotein and homologs of Braun's lipoprotein in other enterobacteriaceae species including S. typhimurium, S. minnesota, S. usumbura (Braun et al., 1970), P. mirabilis (Gruss et al., 1975), M. morganii (Huang et al., 1983), E. amylovora (Yamagata et al., 1981) and S. marcescens (Braun et al., 1970), and in P. aeruginosa (Mizuno and Kageyama, 1979). Like Braun's lipoprotein, these homologs of Braun's lipoprotein are covalently linked to the peptidoglycan through their COOH-terminal lysine residues (Braun et al., 1970; Gruss et al., 1975; Katz et al., 1978; Huang et al., 1983; Mizuno and Kageyama, 1979).

Chemical crosslinking of [^{3}H]leucine-labeled polypeptides in E. coli cell envelope indicates that nine polypeptides including OmpA protein, Braun's lipoprotein, Pal and five other membrane lipoproteins are crosslinked to the peptidoglycan (Leduc et al.,

1989). These five membrane lipoproteins appeared to correspond to the new lipoproteins described by Ichihara et al. (1981). This interesting finding suggests a close association of membrane lipoproteins with the murein layer in E. coli, and this association may play an important role in the structural integrity of the cell envelope in gram-negative bacteria.

Some lipoproteins are enzymes such as penicillinase of B. licheniformis (Neugebauer et al., 1981) and membrane-bound penicillinases of B. cereus and S. aureus (Nielsen and Lampen, 1982), pullulanases of K. pneumoniae and K. aerogenes (d'Enfert et. al, 1987), chitobiase of V. harveyi (Soto-Gil and Zyskind, 1989) and β-1,4-endoglucanase of P. solanacearum (Huang et al., 1989). Lipoproteins can also function as transport proteins (MalX and AmiA of S. pneumoniae, Gilson et. al., 1988), in surface exclusion (TraT of E. coli, Perumal and Minkley, 1984), the release of bacteriocins (Hakkart et al., 1981; Cavard et al., 1985; Cole et al., 1985) or excretion of pullulanase from the outer membrane into the medium (PulS in K. pneumoniae, d'Enfert and Pugsley, 1987).

Braun's lipoprotein is nonessential for the growth and division of E. coli cells, since the deletion of lpp gene is not lethal (Hirota et al., 1977). Lpp-28, one of the new lipoproteins discovered by Ichihara et al., is also nonessential for growth (Yamaguchi and Inouye, 1988). The Pal of E. coli seems to be essential; efforts to isolate null mutations in this gene have not been successful (Chen and Henning, 1987).

V. Purpose of the thesis.

A. Summary of the formation of the bound-form lipoprotein.

The free-form lipoprotein is first synthesized, and subsequently attached to the peptidoglycan. The lipid-modified prolipoprotein from globomycin-treated wild-type strain is covalently linked to the peptidoglycan, while the unmodified prolipoprotein in mutant lppD14 does not form bound-form lipoprotein. The deletion of the COOH-terminal lysine residue prevents the formation of bound-form lipoprotein. The homologs of Braun's lipoprotein in other gram negative bacteria contain a COOH-terminal lysine residue and a positively charged penultimate amino acid residue at their COOH-termini.

The purpose of the thesis is to ascertain what structural features of lipoprotein or prolipoprotein are essential for the formation of murein-bound lipoprotein. More specifically, I would attempt to determine whether or not lipid-modification and processing of prolipoprotein is required for the attachment of lipoprotein to peptidoglycan. I would also use site-directed mutagenesis to systematically alter the COOH-terminal region of the lipoprotein molecule to define the structural features essential for the covalent attachment to peptidoglycan. Finally, I would identify the enzyme responsible for this reaction through the isolation of mutation(s) unlinked to lpp defective in the formation of bound-form lipoprotein.

B. Goals of the thesis.

The goals of this thesis research are as follows:

1. Isolation and characterization of E. coli mutants defective in the formation of murein-bound lipoprotein.

2. Determination of whether the lipid modification/processing of prolipoprotein is a prerequisite for the formation of murein-bound lipoprotein or not.

3. Studies of the effects of charge and secondary structure of the signal sequence on the formation of murein-bound lipoprotein.

4. Studies of the effect of mutational alterations of the COOH-terminal region of the lipoprotein on the covalent attachment of lipoprotein to the murein.

5. Studies of the role of the amino acid sequence internal to the lipoprotein in the formation of bound-form lipoprotein.

C. Plan of the research project.

1. Isolation of E. coli mutants with reduced murein-bound lipoprotein by transposon or nitrosoguanidine mutagenesis. These mutants will be screened for the phenotypes of increased sensitivity to SDS and leakiness of periplasmic RNase. The ratio of the bound- to free-form lipoprotein in these mutants will be measured. Mutations affecting the formation of bound-form lipoprotein will be mapped and characterized.

2. E. coli lpp mutants, D14, R14, L20, T20, V20, G21, A20I23I24, and A20I23K24, which are deficient in lipid modification and/or processing of prolipoprotein, and an E. coli strain producing lipid-deficient OmpF-Lpp hybrid protein will be used to investigate the relationship between prolipoprotein modification/processing and the formation of bound-form lipoprotein.

3. New mutants will be constructed by oligonucleotide-directed mutagenesis to study the effects of the charged residues in the signal sequence of prolipoprotein on the attachment of lipoprotein to the murein.

4. lpp mutants with alterations in the COOH-terminal region of the lipoprotein will be constructed by oligonucleotide-directed mutagenesis, and examined for the formation of murein-bound lipoprotein.

5. An lpp mutant with an internal deletion of twenty-one amino acid residues will be used to study the role of the internal amino acid sequence in the formation of bound-form lipoprotein.

MATERIALS AND METHODS

I. Bacterial strains, phages, and plasmids.

The *E. coli* strains used in this study are listed in Table 1. λTn*10* (*b221 c*I::Tn*10* O_{am} P_{am}) and λ467 (*b221 c*I::Tn*5* O_{am} P_{am}) were obtained from Dr. R. K. Holmes' laboratory of this department. Selected λ clones of the *E. coli* chromosomal gene bank of Kohara (Kohara *et al.*, 1989) were obtained from K. Rudd at FDA (Bethesda, MD) and Y. Kohara at Nagoya University (Nagoya, Japan). M13 mp18 phage used in oligonucleotide-directed mutagenesis was purchased from the BioRad Lab Corp (Richmond, CA). Plasmids pNJ1(*tol*B^+), pNJ111(*exc*C^+), and pNJ301(*exc*D^+) (Lazzaroni *et al.*, 1989) were gifts of J. Lazzaroni at Université Claude Bernard Lyon I (Villeurbanne, France). Plasmid pSKL7f(*tol*A^+) (Levengood and Webster, 1989), pTPS202(*tol*QRAB$^+$) and pTPS304(*tol*RA$^+$) (Sun and Webster, 1986) were gifts from R. Webster of Duke University (Durham, NC). Plasmid vector pINII containing both *lpp* and *lac* promoters (Masui *et al.*, 1983), plasmid pKEN125 (Nakamura *et al.*, 1982), and plasmids containing cloned *lpp* mutations R14 (Gennity *et al.*, 1990), L20, T20, V20 (Pollitt *et al.*, 1986), G21 (Inouye *et al.*, 1983), A20I23I24 and A20I23K24 (Inouye *et al.*, 1986) were gifts from M. Inouye of the University of Medicine and Dentistry of New Jersey (Piscataway, NJ). Plasmid pDOCOO4 containing the *ompF*-*lpp* fusion gene (Choi *et al.*, 1987) was a gift from S. Mizushima at University of Tokyo (Tokyo, Japan).

Table 1. *E. coli* strains used in the present study

Strain	Mating type	Selected genotype	Reference or source
E609	HfrC	*pps*	Yem and Wu (1978)
E610	HfrC	*pps lppD14*	Yem and Wu (1978)
K37	F^-	*galK rpsL* sup^o λ^-	Bachmann (1987)
JE5512	HfrC	*man pps*	Suzuki *et al.* (1976)
JE5505	F^-	*lpo pps his pro gal*	Hirota *et al.* (1977)
CAG5051	HfrH	PO1 *nadA57*::Tn*10* *thi-1*	Singer *et al.* (1989)
CAG5052	Hfr	PO3 *metB1 btuB*::Tn*10*	Singer *et al.* (1989)
CAG5053	Hfr	PO43 *relA1 zbc*::Tn*10*	Singer *et al.* (1989)
CAG5054	Hfr	PO44 *thi-1 trpB83*::Tn*10*	Singer *et al.* (1989)
CAG5055	Hfr	PO45 *thi-1 zed*::Tn*10 supD*	Singer *et al.* (1989)
CAG8209	Hfr	PO13 *thi-1 leu zgh*::Tn*10*	Singer *et al.* (1989)
CAG8160	Hfr	PO86 *leu thi-39*::Tn*10*	Singer *et al.* (1989)
JW381	F^-	*zed-508*::Tn*10 supD*	Wechsler (1971)
NK6033	F^-	*relA1 nadA*::Tn*10*	Wanner (1986)
N3030	F^-	*gal*::Tn*10* λ^-	Lloyd and Buckman (1985)

Table 1. Continued

strain	Mating type	Selected genotype	Reference or source
AB1157	F⁻	galK arg lac xyl his mtl pro leu thr ara supE	Howard-Flanders et al. (1964)
JC3411	F⁻	tolA207	Lazzaroni et al. (1989)
JC3417	F⁻	tolB236	Lazzaroni et al. (1989)
JA221	F'	lpp leuB recA/F'lacI^q	Nakamura et al. (1982)
SD12	F⁻	lppΔ37-57 galK glpD his	Asai et al. (1989)
SD312	F⁻	lppΔ37-57 galK his pgsA	Asai et al. (1989)
CJ236	F⁻	dut ung thi relA	Kunkel (1985)
MV1190	F'	thi supE/F'traD lacI^q	Kunkel (1985)
C600	F⁻	tonA leuB thr supE44	Appleyard (1954)

II. Chemicals and enzymes.

[^{35}S]Methionine (1,000 Ci/mmol), [4,5-^{3}H(N)]leucine (60 Ci/mmol), and [^{14}C]leucine (320 mCi/mmol) were purchased from NEN Du Pont Nuclear Corp (Wilmington, DE). [^{3}H]Palmitic acid (60 Ci/mmol), [^{35}S]dATP and Amplify^{tm} were purchased from Amersham Corp (Arlington Heights, IL). Nitrosoguanidine, isopropyl-1-thio-β-D-galactopyranoside (IPTG), mitomycin C and colicin E1 were purchased from the Sigma Chemical Co (St. Louis, MO). Globomycin was a gift of M. Arai of Sankyo Co (Tokyo, Japan). Muta-gene M13 in vitro mutagenesis kit was purchased from BioRad Lab Corp (Richmond, CA). Restriction enzymes HpaI, SmaI, XbaI, EcoRI and HindIII were purchased from GIBCO and BRL Life Technologies Inc (Gaithersburg, MD). Sequenase Version 2.0 kit was purchased from United States Biochemical Corp (Cleveland, OH). Oligonucleotides were purchased from the oligonucleotide synthesis facility at the Uniformed Services University of the Health Sciences. All other biochemicals and solvents were reagent grade or better. Colicin A, K, E2 and E3 were gifts from Dr. Lazdunski (Paris, France) and also prepared from the colicin producer strains according to the method of Spudich et al. (Spudich et al., 1970).

III. Growth media and culture conditions.

Media used included L broth, M9 glucose minimal medium, M9 glucose medium supplemented with required amino acids (50 μg/ml each) (Maniatis et al., 1982), M63 medium (Silhavy et al., 1984), and 2X YT medium (Miller, 1972). RNase-test plates contained LB

medium plus 1.5% bacto agar, 50 μg/ml toluidine blue, and 0.1% yeast RNA (Quaas et al., 1989). NA-SDS plates contained 2.3% nutrient agar and 0.2% (w/v) SDS (Lopes et al., 1972). All strains were grown at 37°C unless stated otherwise. For growth of strains containing various plasmids, appropriate antibiotics were added to the media at the following concentrations: tetracycline 12.5 μg/ml, ampicillin 50 μg/ml, and kanamycin 50 μg/ml (Maniatis et al., 1982).

IV. Determination of RNase-leaky and SDS-sensitive phenotypes.

Mutants isolated by transposon or nitrosoguanidine mutagenesis or constructed by oligonucleotide-directed mutagenesis were checked for the phenotypes of increased sensitivity to SDS and leakiness of periplasmic RNase as described (Lopes et al., 1972; Quaas et al., 1989). For the RNase-leakiness test, strains were grown on RNase test plates at 37°C overnight. Digestion of the RNA in the media by the RNase leaked from the mutant cells results in a change of the pH of the media which in turn causes the change of toluidine blue to a pink color. Thus a pink halo is seen around each RNase-leaky colony. To determine SDS sensitivity, strains were inoculated on NA-SDS plates, and incubated at 42°C overnight. Mutant cells with increased sensitivity to SDS do not grow on the NA-SDS plates.

V. Ouchterlony, antibiotic, phage, and colicin sensitivity tests.

Ouchterlony double-diffusion tests were carried out on glass

slides coated with 1% Noble agar containing 0.05% SDS in 50 mM sodium barbital buffer (pH 8.6) as described by Wu and Lin (Wu and Lin, 1976). The minimum inhibiting concentration (MIC) of globomycin was determined using microtiter plates. Fifty μl of serial dilutions of the tested antibiotic were added to each well of the microtiter plate. A log-phase culture (A_{600nm}=0.3-0.4) of the tested strain was diluted about 100 times with LB medium, and 50 μl of the diluted culture was mixed with serially diluted antibiotic in the wells. After overnight incubation at 37°C the growth status of the tested strains was checked, and the MIC determined. Phage and colicin sensitivity tests were performed as follows. Ten μl of various dilutions of the phage or colicin were spotted on a lawn of 2×10^8 bacterial cells on L agar plates. Plates were incubated at 37°C overnight. A clear spot was observed with a strain sensitive to the phage or colicin. The dilutions of the phage or colicin which gave clear spots on the bacterial lawn were used to compare the relative sensitivity of bacterial strains to the phage or colicin.

VI. Mutant isolation.

A. Tn*5* and Tn*10* mutagenesis.

Transposons containing kanamycin-resistant (Km^r) or tetracycline-resistant (Tc^r) gene have been used successfully in the isolation of null mutations in the target genes in *E. coli*. Standard techniques for transposon mutagenesis were used as described (Silhavy *et al.*, 1984). One ml of a mid-log phase

culture of the wild-type E. coli strain was mixed with 50 μl of a high-titer lysate (>10^{10}/ml) of λTn10 or λ467 (cI::Tn5) at a multiplicity of infection of 5-10. After incubation at room temperature for 5 min, one ml of LB containing 40 mM sodium citrate (pH 7.0) was added to the tube, and the mixture was incubated with shaking for 1 hr at 37°C. The cells were collected by centrifugation, and plated on LB agar containing 12.5 μg/ml tetracycline (for Tn10) or 50 μg/ml kanamycin (for Tn5). Km^r or Tc^r mutants were screened for RNase-leakiness and SDS^s phenotypes as described above. Mutants with phenotypes of increased sensitivity to SDS and leakiness of RNase were checked for the ratio of the bound- to free-form lipoprotein by the method described on pages 38-42 of this thesis.

B. Nitrosoguanidine mutagenesis.

In addition to transposon mutagenesis, chemical mutagenesis with nitrosoguanidine was also used in the present study for the isolation of E. coli mutants defective in the formation of bound-form lipoprotein. This would allow the isolation of partially defective mutants or conditionally lethal mutants. Two ml of late-log phase culture of wild-type strain E609 or K37 were mixed with 50 μl of nitrosoguanidine (2.5 mg/ml in 95% ethanol) and incubated for 10 or 15 min at 37°C. After washing with 2 ml of M63 medium, the cells were resuspended in 2 ml LB and incubated for 3-4 hrs at 37°C. One tenth ml of 10^{-1} and 10^{-2} dilutions of the culture were plated on LB agar and incubated at 37°C overnight. The colonies

were replica-plated on RNase-test plates and NA-SDS plates, and RNase-lky and SDS^s colonies were analyzed for the ratio of the bound- to free-form lipoprotein.

C. Construction of E. coli lpp mutants by oligonucleotide-directed mutagenesis.

1. Mutagenesis procedure.

Oligonucleotide-directed mutagenesis was carried out using single-stranded M13 DNA containing the lpp gene as the template according to the method of Kunkel (1985). The procedures for the oligonucleotide-directed mutagenesis and subcloning of the mutant lpp alleles are shown in Fig. 6. M13 lpp phage was constructed by subcloning the lpp structural gene from the plasmid pJG02 (Giam et al., 1984a) to M13 mp18 phage. Plasmid pJG02 DNA was digested with restriction enzymes XbaI and HpaI, and the RF DNA of M13 mp18 was digested with XbaI and SmaI. After electrophoresis of the restriction enzyme digested samples in low-melting agarose gel, the gel bands containing the XbaI-HpaI cleaved pJG02 DNA and the XbaI-SmaI cleaved M13 vector were cut and mixed at 70°C. The DNA fragments of the lpp gene and the M13 mp18 vector were ligated with T4 DNA ligase at 20°C overnight, and the ligation product was used to transfect strain CJ236 dut ung. Uracil-containing M13 (U-M13) lpp phages were precipitated by PEG8000, and the single-stranded M13 lpp DNA was extracted from the M13 lpp phages with phenol-chloroform. Phosphorylated oligonucleotides containing the designed mutations were annealed with the purified U-M13 lpp

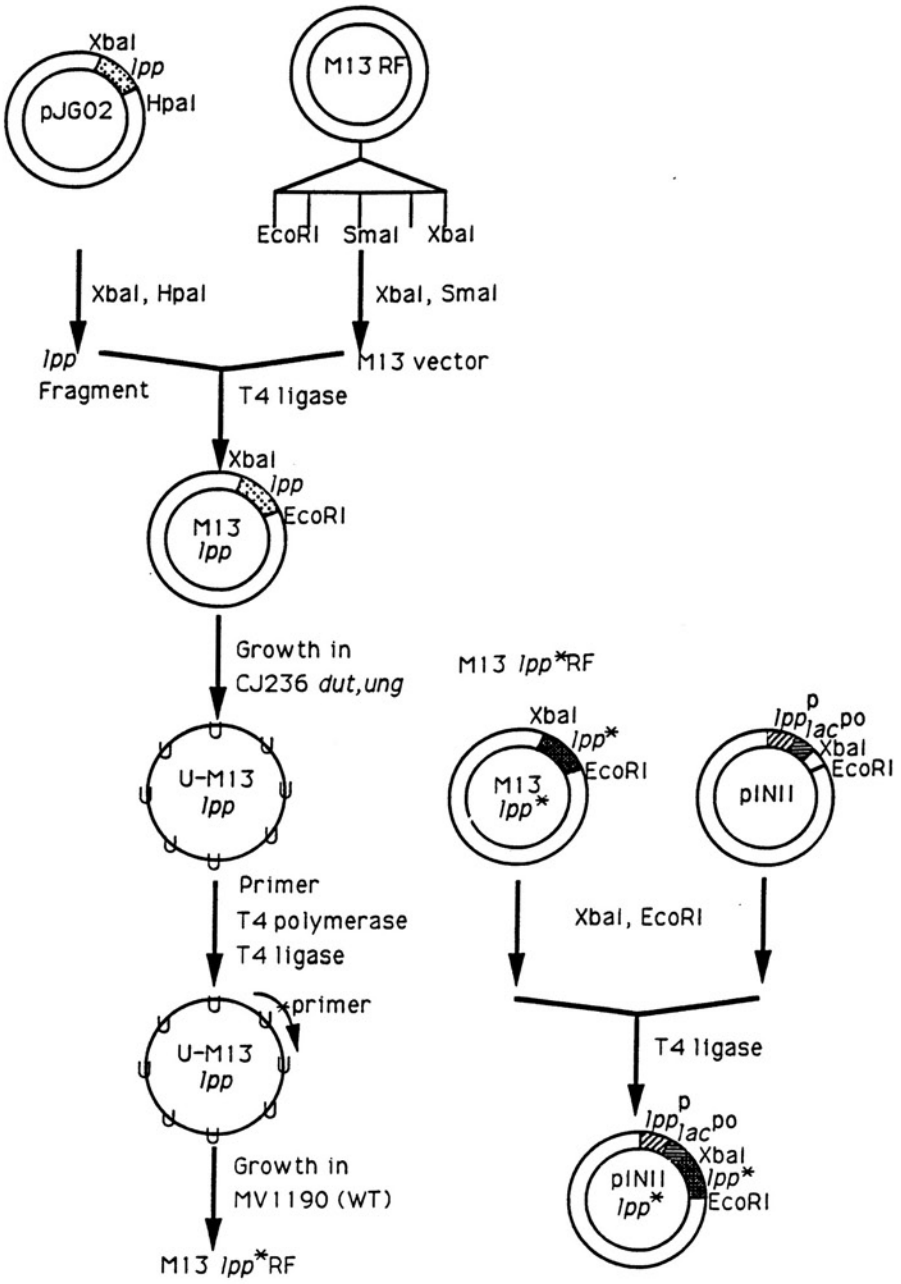


Fig. 6. Construction of lpp mutations by oligonucleotide-directed mutagenesis. The wild-type lpp gene was subcloned from plasmid pJG02 to M13 phage. The uracil containing single-stranded DNA of the recombinant M13 phage (U-M13 lpp) was used as the template in oligonucleotide-directed mutagenesis. The constructed mutant lpp alleles (lpp*) were subcloned into the plasmid vector pINII which contained both lpp and lac promoters.

single-stranded DNA by incubation of the samples at 70°C for one min and cooling to 30°C at a rate of 1°C/min. Synthesis of the complementary DNA strand was initiated by the addition of 0.4 mM each dNTP, 0.75 mM ATP, 1 unit T4 DNA polymerase and 5 units T4 DNA ligase, and the sample was incubated at 37°C for 90 min. Wild-type strain MV1190 was transfected with the *in vitro* synthesized RF DNA of M13 *lpp* to obtain single plaques. Mutant plaques containing the desired mutations were identified by DNA sequence analysis.

2. Subcloning of the mutant *lpp* alleles.

The mutant *lpp* alleles constructed by oligonucleotide-directed mutagenesis were subcloned into an expression vector, pINII, which contains both the *lac* and *lpp* promoters (Fig. 6). Both the RF DNA of the M13 *lpp* mutants and the pINII plasmid DNA were digested with *Xba*I and *EcoR*I at 37°C for 2 hrs. Following agarose gel electrophoresis, the *Xba*I-*EcoR*I fragments containing the *lpp* mutant alleles and the pINII vectors were isolated and ligated with T4 DNA ligase at 15°C overnight. An *E. coli* K-12 strain, JA221 *lpp*$^-$ /F'*lacI*^{q} (Nakamura *et al.*, 1982) was used as the host for the cloned mutant *lpp* genes. The *lpp* mutation was confirmed by DNA sequence analysis of the double-stranded plasmid DNA from the Lpp$^+$ transformants.

VII. Quantitation of free- and bound-form lipoprotein in *E. coli*.

A. Determination of the bound-form lipoprotein content in the wild-type and mutant strains.

1. Single-labeling protocol.

Two tenth ml of an overnight culture of E. coli K-12 wild-type or mutant strain in M9 medium supplemented with 0.2% glucose, required amino acids, methionine (10 μg/ml) and appropriate antibiotics was used to inoculate a 5 ml culture in the same medium. The cells were incubated at 37°C until A_{600nm} reached 0.2. When indicated, globomycin and IPTG were added to a final concentration of 80 μg/ml and 3 mM, respectively, 20 min prior to labeling. Fifty μCi of [^{35}S]methionine was added to the culture, and incubation continued until A_{600nm} reached 1.0. The cells were harvested by centrifugation at 5,000 x g for 15 min at 4°C, resuspended in 5 ml of 10 mM sodium phosphate buffer (pH 7.0) and broken by sonication. The cell envelope was isolated by centrifugation at 200,000 x g for 60 min at 4°C, and extracted with 500 μl of 1% SDS in 10 mM sodium phosphate buffer (pH 7.0) at 100°C for 20 min. Free-form lipoprotein was isolated from the 1% SDS soluble fraction of the cell envelope by a modified immunoprecipitation procedure of Giam et al. (1984a). One tenth ml of the 1% SDS soluble fraction was diluted with 1 ml of 10 mM sodium phosphate buffer (pH 7.0), mixed with 60 μl of fixed Staphylococcus aureus cells, incubated at 4°C for 15 min, and centrifugation for 5 min in a microfuge to remove the nonspecific proteins bound to the fixed S. aureus cells. The supernatant was transferred to a microfuge tube, mixed with 50 μl of antilipoprotein serum, and incubated at 37°C for 2 hrs or at 4°C overnight. After the addition of 60 μl of S. aureus cell

suspension, the mixture was incubated at 4°C for an additional 15 min. The immunoprecipitate was then centrifuged, washed first with 1 ml of NET-NP40 buffer containing 150 mM NaCl, 50 mM EDTA, 50 mM Tris-HCl (pH. 7.5) and 0.05% NP-40, and then with 1 ml of 10 mM sodium phosphate buffer (pH 7.0). The immunoprecipitate was dissolved in 50 μl Laemmli gel sample buffer (Laemmli, 1970).

For the isolation of the bound-form lipoprotein, the SDS-insoluble pellet was reextracted with 1 ml of 4% SDS in 10 mM sodium phosphate buffer (pH 7.0), with unlabeled cell envelope added as the carrier. After centrifugation at 200,000 x g for 90 min, the pellet was washed with 8 ml of water three times. The murein sacculus containing the bound-form lipoprotein was resuspended in 200 μl of 10 mM sodium phosphate buffer (pH 7.0), digested with lysozyme (300 μg/ml) overnight at 37°C, lyophilized, and dissolved in 50 μl of Laemmli sample buffer.

2. Double-labeling protocol.

A double labeling protocol was also used to compare the relative amount of murein-bound lipoprotein in the mutant (MT) with that in the wild-type (WT) strain. M9 medium supplemented with 0.2% glucose, required amino acids, leucine (10 μg/ml) and appropriate antibiotics was used for the labeling of cells. Thirty μCi [^{3}H]leucine was added to a 5 ml culture of each of the mutant strains, and 100 μCi [^{14}C]leucine was added to a 30 ml culture of the wild-type strain. Following solubilization of labeled cell envelope with 1% SDS, the counts of [^{3}H]leucine-labeled mutant cell

envelope were matched with the corresponding counts of [^{14}C]leucine-labeled wild-type cell envelope to give a ^{3}H/^{14}C ratio of about 3. The mixture of [^{3}H]leucine-labeled mutant cell envelope and [^{14}C]leucine-labeled wild-type cell envelope was centrifuged at 200,000 x g for 90 min. Free-form and bound-form lipoproteins were isolated from the mixed cell envelope by the procedure described for the single-labeling protocol. The ^{3}H/^{14}C ratios of the free- and bound-form lipoproteins were determined by liquid scintillation counting.

3. Quantitation of the free- and bound-form lipoproteins.

The [^{35}S]methionine radioactivity or the ^{3}H/^{14}C ratio in the free- and bound-form lipoproteins were determined by liquid scintillation counting, and the ratio of bound- to free-form lipoprotein was calculated. In the double-labeling protocol, the relative content of the bound-form lipoprotein in the mutant strain was estimated by normalizing the ^{3}H/^{14}C ratio of the bound-form with that of the free-form lipoprotein as follows:

$$\frac{[^{3}H]\ MT\ BF}{[^{14}C]\ WT\ BF} \Big/ \frac{[^{3}H]\ MT\ FF}{[^{14}C]\ WT\ FF} = \frac{[^{3}H]\ MT\ BF/[^{3}H]\ MT\ FF}{[^{14}C]\ WT\ BF/[^{14}C]WT\ FF} \quad (1),$$

and

$$\frac{[^{3}H]\ WT\ BF}{[^{14}C]\ WT\ BF} \Big/ \frac{[^{3}H]\ WT\ FF}{[^{14}C]\ WT\ FF} = \frac{[^{3}H]\ WT\ BF/[^{3}H]\ WT\ FF}{[^{14}C]\ WT\ BF/[^{14}C]WT\ FF} = 1 \quad (2).$$

The ratio of bound- to free-form lipoprotein in wild-type strain

is approximately 0.5 regardless whether the cells are labeled with [^{3}H] or [^{14}C]leucine. Accordingly, the ratio of the ^{3}H/^{14}C of the bound-form lipoprotein to that of the free-form lipoprotein is equal to one for the wild-type strain (Equation 2). Equation 1 stipulates that the ratio of ^{3}H/^{14}C ratio of the bound-form lipoprotein to that of the free-form lipoprotein will approximate the relative content of the bound-form lipoprotein in the [^{3}H]leucine-labeled mutant to that in the [^{14}C]leucine-labeled wild-type strain.

B. Analysis of the free- and bound-form lipoproteins by SDS-polyacrylamide gel electrophoresis.

Two SDS-polyacrylamide gel systems, the Ito gel (Ito et al., 1980) and the Tricine-SDS gel (Schägger and Jagow, 1987), were used for the analysis of the free- and bound-form lipoproteins in the wild-type and mutant strains. The Ito gel system was used to separate the prolipoprotein (lipid-modified or unmodified) from the mature free-form lipoprotein. The Tricine-SDS gel system was used to separate the lipid-modified prolipoprotein from the unmodified prolipoprotein.

1. Ito gel system.

In this gel system, a 19.3% polyacrylamide separating gel containing 6 M urea was used. The gel was run at 20 mA for 3-4 hrs until the blue-dye front reached the bottom of the gel. The gel was then stained with a solution containing 25% isopropanol, 10%

acetic acid and 0.25% Coomassie brilliant blue (R250) for 30 min, and destained with a solution containing 10% acetic acid and 12.5% methanol until the protein bands in the gel became clearly visible. After treatment with Amplify^{tm} for 20 min, the gel was dried under vacuum, and exposed to a Kodak X-AR5 film.

2. Tricine-SDS gel system.

The separating gel of the Tricine-SDS gel system contained 16.5% acrylamide, 1% bisacrylamide and 13% glycerol. The gel was run at 30 V for about one hr until all samples entered the gel, and at 105 V for 18-20 hrs. The procedures for the staining, destaining and drying of the gel, and fluorography were the same as described above.

VIII. Genetic and DNA techniques.

A. Mapping of mutations.

1. Conjugation.

E. coli Hfr strains containing Tn10 insertions at known locations were used as the donors in mapping of the mutations by conjugation. Conjugation was carried out as described by Miller (1972). Overnight cultures of the Hfr donors were diluted 1:50 in LB medium and allowed to grow at 37°C until A_{600nm}=0.2 - 0.3. Half an ml of each of the Hfr cultures was mixed with 4 ml of an exponentially growing culture (2-5 x 10^8 cells/ml) of the streptomycin-resistant recipient in a 125 ml flask, and incubated in a slowly shaking waterbath at 37°C for 90 min. One tenth ml of

10^{-1} and a 10^{-2} dilutions of the mating mixture were plated on the selective plates containing 12.5 μg/ml tetracycline and 100 μg/ml streptomycin, and the plates were incubated at 37°C overnight. The transconjugants were purified on the same selective plates, and screened for the phenotypes of the unselected markers (RNase-leakiness and SDS sensitivity).

F' transfer was carried out using either the liquid culture mating or the spot mating method as described by Miller (1972). Equal volumes (0.5 ml) of both the donor and recipient strains were mixed, and conjugation was carried out as described for the Hfr mating. For the spot mating, a M9 minimal plate with appropriate supplementation was divided into three sections. A drop of an exponential culture of the donor was applied with a Pasteur pipette to section one, while a drop of the recipient was spotted on section two. Both the donor and the recipient were spotted together on the third section. After the spots were dry, the plates were incubated at 37°C overnight. Transconjugants which grew in the spot where both the donor and recipient were applied were purified and tested for the unselected phenotypes.

2. P1 transduction.

(1) Preparation of P1 lysates.

Fifty μl of an overnight culture of the donor strain in LB medium was inoculated in 5 ml of LB medium containing 0.2% glucose and 5 mM $CaCl_2$, and incubated for 30 min at 37°C with aeration. One tenth ml of a P1<u>vir</u> lysate (5 x 10^8 phages/ml) was added, and the

culture was continually shaken for 2-3 hrs until lysis. One tenth ml chloroform was added to the lysate, vortexed, and centrifuged at 4,500 x g for 10 min; the supernatant was transferred to a tube containing 0.1 ml chloroform and stored at 4°C.

(2) Transduction with P1 lysates.

An overnight culture of the recipient strain in LB medium was centrifuged at 4,500 x g for 10 min, and resuspended in 2.5 ml of sterilized distilled water containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$. One tenth ml aliquots of the recipient cells were added to each of 4 tubes containing 0, 10, 50 or 100 μl of P1 lysate, respectively. The mixtures were votexed and incubated for 30 min at 37°C without shaking. One tenth ml of 1 M sodium citrate (pH 7.0) and 1 ml of LB medium were added to each tube. After shaking for 1 hr at 37°C, the cells were pelleted by centrifugation and plated on the selective medium. After overnight incubation at 37°C, 100 to 200 transductant colonies would appear on the selective plates. The transductants were purified and screened for the unselected markers.

3. Transformation.

The transformation procedure described by Silhavy et al. (1984) was used. Competent cells of the host strain were prepared by the $CaCl_2$ method. Twenty-five ml LB medium in a 125 ml flask was inoculated with 0.1 ml of an overnight culture of the host strain, and incubated at 37°C for 2-3 hrs until A_{600nm}=0.3. The

cells was centrifuged at 5,000 x g for 10 min at 4°C, resuspended in 12.5 ml of 100 mM $CaCl_2$, and incubated on ice for 20 min. After centrifugation, the cells were resuspended in 2.5 ml of 100 mM $CaCl_2$. One hundred and fifty µl aliquots of the competent cells were distributed into sterile microfuge tubes placed in an icebath. Three to five µl of plasmid DNA (about 0.5-1.0 µg plasmid DNA) or 10-15 µl of ligation mixtures were added to the competent cells, and the mixtures were incubated on ice for 60 min and heat shocked at 42° for 2 min. One ml of LB medium was added, and the cells were incubated at 37°C for 60 min, centrifuged, and plated on LB agar plates containing appropriate antibiotics. The plates were incubated at 37° overnight, and the transformants were purified on the same selective media.

B. DNA techniques.

1. DNA extraction.

Plasmid DNA and RF DNA of M13 phage were extracted using an alkali-mini lysate method described by Silhavy et al. (1984). The strain harboring the plasmid was inoculated in 5 ml of LB medium containing appropriate antibiotics, and incubated at 37° overnight. The cells were centrifuged at 4,500 x g for 15 min, and resuspended in 0.2 ml of lysis buffer containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), and 5 mg/ml lysozyme. After incubation on ice for 5 min, 0.4 ml of 0.2 N NaOH-1% SDS was added, and the sample was incubated on ice for another 5 min. Three tenths ml of 5 M potassium acetate (pH 4.8) was added, and the sample was

centrifuged at 4,500 x g for 15 min. The supernatant was transferred to a new tube, and 50 μg of RNase A was added. After incubation at 37°C for 30 min, the sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol mixture (v/v 25:24:1) twice. The top aqueous layer was collected from the tube, and two volumes of ice-cold ethanol was added. After incubation in a dry ice-ethanol bath for 20 min, the sample was centrifuged in a microfuge at 4°C for 15 min to collect the DNA precipitate. The supernatant was removed, and the DNA pellet was washed with 1 ml of 70% ethanol, dried under vacuum, and resuspended in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Five μl of the DNA solution containing 1 to 2 μg plasmid DNA was used for restriction enzyme analysis.

Single-stranded M13 DNA was prepared as follows. A single M13 lpp plaque was transferred from the plate to 2 ml of 2X YT medium containing 1:100 dilution of an exponentially growing culture of the host cells. The culture was incubated at 37°C with shaking for 5-8 hrs, and centrifuged for 10 min in a microfuge. The phage-containing supernatant was transferred to a new tube, and 1/9 volume of 40% PEG-8000 and 1/9 volume of 5 M sodium acetate (pH 7.0) were added. The sample was incubated at 4°C for 15 min, and centrifuged for 10 min in a microfuge. The supernatant was removed, and the pellet was resuspended in 0.2 ml of TE buffer and extracted with 0.2 ml of phenol:chloroform (v/v 3:1) twice. The single-stranded M13 DNA was precipitated with ethanol and dried under vacuum.

2. Restriction enzyme digestion.

Restriction enzyme digestion of DNA was performed as described by Maniatis et al.(1982). Two and half μl of 10X buffer supplied by the manufacturers, 1 μg DNA, 10 units of the restriction enzyme, and distilled water to a final volume of 25 μl were added sequentially to a 1.5 ml sterile microfuge tube. The sample was mixed and incubated at 37° for 2 hrs. Five μl of 6X sample buffer containing 20% Ficoll and 0.05% bromophenol blue was added, and the sample was loaded to a 0.7% agarose gel. The agarose gel electrophoresis was carried out at 70 V for 2-4 hrs, and the gel was stained with 0.5 μg/ml ethidium bromide for 10 min, and photographed using UV light.

3. Sequencing.

DNA sequencing was carried out using a Sequenase Version 2.0 kit and the protocol provided by the manufacturer. The annealing reaction was carried out in a microfuge tube containing 100 nmol of primer, 2 μl of 5X reaction buffer, and 7 μl of DNA (approximately 1 μg of M13 single-stranded DNA or 3-5 μg of NaOH-denatured plasmid DNA). The tube was heated to 65°C for 2 min, then cooled slowly to 30°C over a period of about 40 min. Once the temperature was below 30°C, annealing was complete and the tubes were placed on ice. One μl of 0.1 M DTT, 2 μl of a labeling mixture containing 1.5 μM each of dGTP, dCTP and dTTP, and 20 μCi of [α-^{35}S]dATP were added to the reaction mixture, followed by the addition of 2 μl of diluted Sequenase Version 2.0. The contents in each tube were mixed

thoroughly and incubated at the room temperature for 2-5 min. Two and half μl of the ddATP, ddGTP, ddTTP or ddCTP termination mixtures was added to each of 4 marked tubes, and prewarmed at least 1 min at 37°C. The labeling reaction mixture was divided equally into these 4 tubes containing the termination mixtures, and incubated at 37°C for 3-5 min. Four μl of a stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF was added to each tube, and the samples were heated at 75°C for 2 min, and loaded onto a 6% polyacrylamide gel containing 8M urea. The sequence gel was prewarmed for 30 min, and run for 3-4 hrs at 65 W. After fixing in a solution containing 10% acetic acid and 12% methanol for 40 min, the gel was dried and used for autoradiography with Kodak XR-5 films.

RESULTS

I. RNase-leaky mutants with reduced bound-form lipoprotein.

A. Mutants isolated by transposon mutagenesis.

1. Tn*10* insertion mutant.

RNase-leaky and SDS-sensitive phenotypes were used to identify potential mutants with reduced amount of bound-form lipoprotein since all known mutants with no or greatly reduced amount of bound-form lipoprotein exhibited these phenotypes (Hirota *et al.*, 1977; Weigand *et al.*, 1976; Lin *et al.*, 1978). Forty-four RNase-leaky and SDS-sensitive colonies were isolated from about three thousand Tc^r colonies following Tn*10* mutagenesis of *E. coli* wild-type strain E609, and one of these mutants, strain E30, was found to contain no lipoprotein by the Ouchterlony test. The Tn*10* insertion in strain E30 was found to be in the *lpp* structural gene, based on P1 transduction (Table 2); the *lpp* gene in *E. coli* is known to be linked to *man* and *pps* with a cotransduction frequency of about 10% for each marker (Hirota *et al.*, 1977). The remaining 43 RNase-leaky and SDS^s Tc^r mutants were found to contain nearly normal amount of bound-form lipoprotein, and were not further studied.

2. Tn*5* mutant producing a truncated lipoprotein.

Seventy-two mutants with the phenotypes of increased sensitivity to SDS and leakiness of RNase were isolated from about four thousand Km^r colonies. One Tn*5* insertion mutant, strain K20, was identified as having greatly reduced amounts (less than 3% of

Table 2. Mapping of the Tn*10* insertion in strain E30 and the Tn*5* insertion in strain K20 by P1 transduction.

Donor strain	Recipient strain	Selected marker	Unselected marker	Cotransduction frequency
E30(*lpp*::Tn*10*)	JE5512(*man pps*)	Tc^r	*man*$^+$	12%
		Tc^r	*pps*$^+$	14%
K20(*lpp*::Tn*5*)	JE5505(*lpp*)	Km^r	*lpp*$^+$	100%
E30(*lpp*::Tn*10*)	K20(*lpp*::Tn*5*)	Tc^r	Km^s *lpp*	100%

Strain E30 and JE5505 were negative for Lpp based on Ouchterlony test whereas strain K20 was positive for Lpp based on Ouchterlony test, even though both E30 and K20 contained a transposon insertion in the *lpp* gene.

the wild-type level) of the bound-form lipoprotein, but apparently normal amounts of the free-form lipoprotein based on the results of a double-labeling experiment. P1 transduction experiments with K20 as the donor and JE5505 (*lpp*) as the recipient, and with E30 (*lpp*::Tn*10*) as the donor and K20 as the recipient clearly indicated that the Tn*5* insertion in strain K20, the Tn*10* insertion in strain E30, and the *lpp* deletion in JE5505 were all allelic (Table 2). However, mutant K20 still contained lipoprotein cross-reacting polypeptide based on a positive Ouchterlony test using anti-lipoprotein serum. Radiochemical studies revealed that mutant K20 (*lpp*::Tn5) synthesized a truncated lipoprotein. The precursor form of the truncated lipoprotein was detected by SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled lipoprotein from globomycin-treated mutant cells. The size of the truncated lipoprotein in mutant K20 was smaller than that of the normal mature free-form lipoprotein (Fig. 7). These results suggest that Tn*5* insertion in the *lpp* gene resulted in the formation of a truncated lipoprotein approximately 4-5 kD in apparent molecular weight. Since the truncated lipoprotein presumably lacked the COOH-terminal region of the normal lipoprotein, including the Lys78, it was not covalently attached to the peptidoglycan.

B. Mutants isolated by chemical mutagenesis.

1. Isolation of mutants.

One hundred and sixteen RNase-leaky and SDS-sensitive mutants were isolated following nitrosoguanidine mutagenesis (62.5 μg/ml,

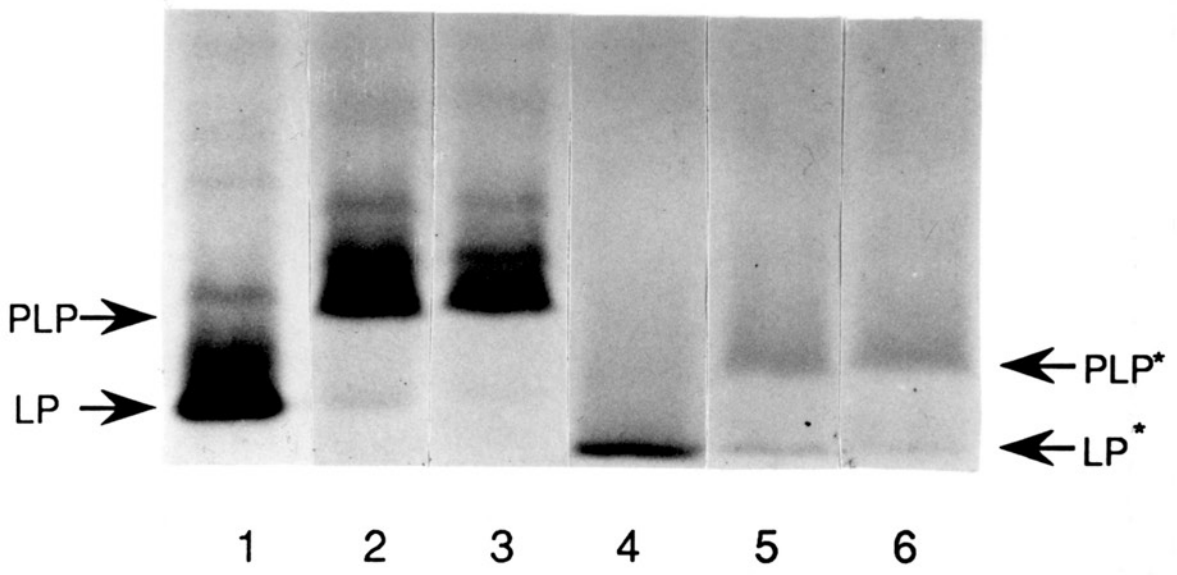


Fig. 7. SDS-polyacrylamide gel electrophoresis of truncated lipoprotein encoded by lpp::Tn5 in mutant strain K20. E. coli cells of the wild-type strain E609 and mutant K20 were labeled with [^{35}S]methionine. Globomycin was added to final concentrations of 0, 50 and 80 μg/ml, respectively, 20 min prior to the labeling. The procedures of [^{35}S]methionine labeling, immunoprecipitation and SDS-PAGE using the Ito gel system were described in **MATERIALS AND METHODS**. Ten μl of the free-form lipoproteins were loaded in each lane of the gel. Lanes 1-3, wild-type strain E609 with 0, 50 and 80 μg/ml globomycin, respectively. Lanes 4-6, mutant strain K20 with 0, 50 and 80 μg/ml globomycin, respectively. PLP: prolipoprotein; LP: mature free-form lipoprotein; PLP*: truncated mutant prolipoprotein; LP*: truncated mutant lipoprotein.

10-15 min at 37°C), and three mutants (K3, K86, and MTA) were found to have a reduced ratio of bound-form to free-form lipoprotein as compared to the parental strains (K37 was the parental strain for K3 and K86, and E609 was the parental strain for MTA [Table 1]). These three mutants contained about 20-50% of the bound-form lipoprotein present in their respective wild-type parental strains (Table 3 and Fig. 8).

2. Mapping of lky mutations in strains K3 and K86.

The lky mutations in strains K3 and K86 were mapped by conjugation, F' transfer and transduction. A Tn10 Hfr kit consisting of seven Hfr strains with different origins of transfer and known locations of the Tn10 insertions (Singer et al., 1989) was used to map the lky mutations in strains K3 and K86. Based on the results of this rapid mapping technique, the lky mutation in strain K3 was located between 12 min (zbc::Tn10) and 17 min (nadA::Tn10) on the E. coli chromosome (data not shown). The results of the mapping of the lky mutation in strain K86 were more complex. Like mutant K3, conjugation of mutant K86 with Hfr strains containing zbc::Tn10 or nadA::Tn10 gave wild-type recombinants with RNase-nonleaky and SDS^r phenotypes. However, Hfr strain CAG5055 with an origin of transfer at 61 min and Tn10 insertion at 43 min was also able to yield recombinants of mutant K86 with the wild-type phenotype. These results may be explained in two ways: strain K86 might be a double mutant with one mutation at 12-17 min and a second mutation at 40-50 min, and the RNase-

Table 3. Contents of murein-bound lipoprotein in mutant strains K3, K86 and MTA

Strain	BF lipoprotein (percent of wild-type)	
	double labeling	single labeling
K37(wild-type)	100	100
E609(wild-type)	100	100
E610(lppD14)	10	7
K3	50	48
K86	39	30
MTA	25	20

The procedures for single- and double-labeling of E. coli cells are described in the **MATERIALS AND METHODS.** The lppD14 mutant strain E610 containing a structurally altered prolipoprotein (Gly14 to Asp14) which is not modified nor covalently attached to the peptidoglycan (Lin et al., 1978) was used as a control in measuring the contents of murein-bound lipoprotein. The content of murein-bound lipoprotein in the wild-type strain was set as 100%.

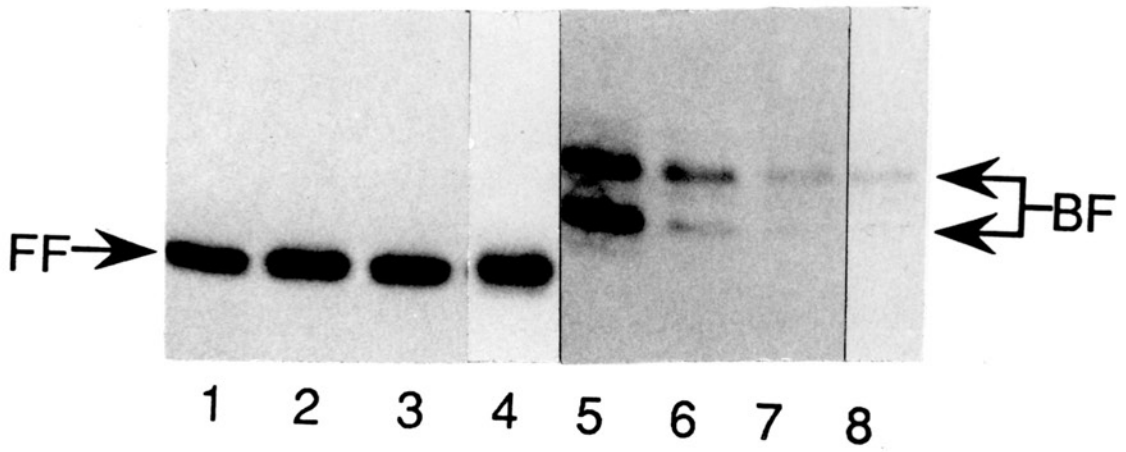


Fig. 8. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from strains K3, K86 and MTA. The procedures of [^{35}S]methionine labeling of the wild-type and mutant strains and SDS-PAGE of the free- and bound-form lipoprotein for this and all the subsequent experiments were described on pages 38-43. Equal volumes (5 μl) of the lipoprotein samples (50 μl) from 5 ml cultures were loaded in each lane of a Tricine-SDS gel. Lanes 1-4, free-form lipoproteins from the wild-type strain K37, and mutant strains K3, K86 and MTA, respectively; lanes 5-8, bound-form lipoproteins from the wild-type strain K37, and mutant strains K3, K86 and MTA, respectively. FF: free-form lipoprotein; BF: bound-form lipoprotein.

leaky and SDS-sensitive phenotypes required the presence of both mutations. Alternatively, strain CAG5055 might transfer a suppressor at 40-50 min for a mutation located at 12-17 min in mutant K86. Results shown in a later section indicated that the latter possibility was true.

Four F' strains which contained overlapping regions of the E. coli chromosome at 17 min (Low, 1972) were used to further map the lky mutations in strains K3 and K86. lky mutations in K3 and K86 were complemented by F' from strain 5104, 5549 and 4253 but not by F' from strain 4287 (Fig. 9). It is clear that the lky mutations in mutants K3 and K86 are located at 17 minute of the E. coli genetic map.

P1 transduction indicated that the lky mutations in strains K3 and K86 were closely linked to nadA (96%) and to gal (73%). In addition, data from a three-factor cross placed the lky mutations in K3 and K86 between nadA and gal (Table 4).

3. The nature of K86 lky mutation.

Using nadA::Tn10 as the selected marker, the lky mutations in both strains K3 and K86 were transduced into the wild-type strain K37. More than ninety percent of the Tc^r transductants of K37 with either K3 lky nadA::Tn10 or K86 lky nadA::Tn10 as the donor showed RNase-leaky and SDS^s phenotypes (Table 4). These results indicate that a mutation linked to nadA in mutant K86 is sufficient to confer the RNase leaky and SDS^s phenotypes on K37 strain. Thus the data from the conjugation experiment described above cannot be

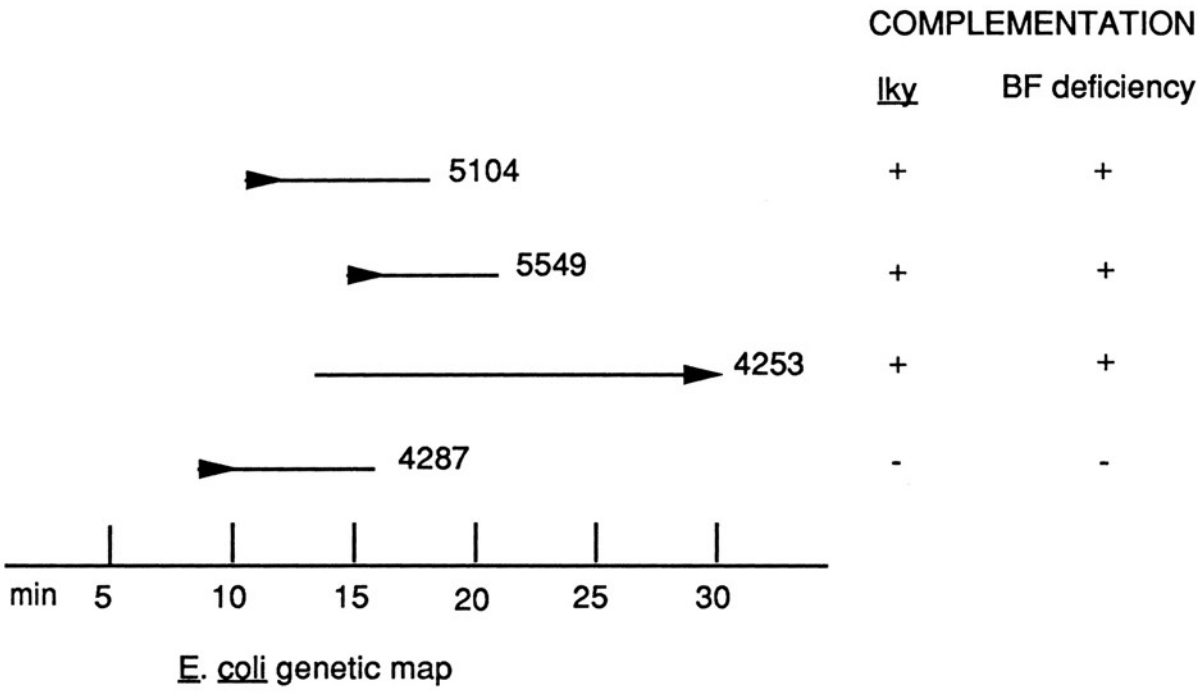


Fig. 9. Complementation of the lky and bound-form (BF) lipoprotein defect in strains K3 and K86 by E. coli F' factors. The genetic maps of the four F' factors in strains 4253, 4287, 5104 and 5549 with overlapping regions at 17 min of the E. coli chromosome are shown. The arrows indicate the direction and origin of the transfer of the F' factors.

Table 4. Mapping of *lky* mutations in strains K3, K86, and MTA by P1 transduction.

Donor strain	Recipient strain	Selected marker	Unselected marker	Cotransduction frequency
NK6033(*nadA*::Tn*10*)	K3 (*lky*)	*nadA*::Tn*10*	*lky*$^+$	96%
NK6033(*nadA*::Tn*10*)	K86(*lky*)	*nadA*::Tn*10*	*lky*$^+$	97%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K3 (*lky galK*)	*nadA*::Tn*10*	*lky*$^+$ *gal*$^+$	68%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K3 (*lky galK*)	*nadA*::Tn*10*	*lky*$^+$ *gal*$^-$	28%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K3 (*lky galK*)	*nadA*::Tn*10*	*lky gal*$^+$	0%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K3 (*lky galK*)	*nadA*::Tn*10*	*lky gal*$^-$	4%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K86(*lky galK*)	*nadA*::Tn*10*	*lky*$^+$ *gal*$^+$	65%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K86(*lky galK*)	*nadA*::Tn*10*	*lky*$^+$ *gal*$^-$	33%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K86(*lky galK*)	*nadA*::Tn*10*	*lky gal*$^+$	0%
NK6033(*nadA*::Tn*10* *gal*$^+$)	K86(*lky galK*)	*nadA*::Tn*10*	*lky gal*$^-$	2%
N3030(*gal*::Tn*10* *lky*$^+$)	K3 (*lky galK*)	*gal*::Tn*10*	*lky*$^+$	72%
N3030(*gal*::Tn*10* *lky*$^+$)	K86(*lky galK*)	*gal*::Tn*10*	*lky*$^+$	74%
18495(*zih*::Tn*10*)	MTA(*lky*)	*zih*::Tn*10*	*lky*$^+$	53%
18496(*fad*::Tn*10*)	MTA(*lky*)	*fad*::Tn*10*	*lky*$^+$	96%

attributed to K86 being a double mutant with a second mutation at 40-50 min also required for its lky phenotype. To determine which gene(s) at 40-50 min of the E. coli genome may suppress the lky mutation in mutant K86, a series of E. coli strains containing Tn10 insertions in genes between 40 and 50 min were used as donors in P1 transduction of strain K86. Of nine strains used as the donors, only the Tc^r transductants obtained with JW381 zed::Tn10 as the donor showed the RNase-nonleaky phenotype at a cotransduction frequency of 79%. A closer examination of the genotypes of the Hfr strain CAG5055 and strain JW381 revealed that both strains contained a supD allele which is linked to zed::Tn10 at a cotransduction frequency of 80%. Thus the lky mutation in strain K86 is most likely an amber mutation which could be suppressed by supD. To confirm this hypothesis, the lky mutation in K86 was transduced into both sup^+ recipients (AB1157 and C600) and sup^o recipient (K37) using nadA::Tn10 as the selected marker. None of the Tet^r transductants of sup^+ recipients was RNase-leaky, while most of the Tet^r transductants of the sup^o recipient were RNase-leaky (data not shown). These data confirmed that the lky mutation in mutant K86 was an amber mutation.

4. Complementation of the lky mutations in strains K3 and K86.

Kohara E. coli chromosomal gene bank in λ phage was used to complement the lky mutations in K3 and K86. Based on the close linkage of the lky mutations to nadA, we chose three λ phage clones (λ176, λ178 and λ179) for the complementation test. The lky

mutations in both K3 and K86 were complemented by λ178 (also called 10G5) but not by λ176 (7E10) or λ179(4H1). However, none of these λ clones complemented the deficiency in the formation of bound-form lipoprotein in strains K3 and K86. λ clone 10G5 had a 15 kilobase insertion containing genes located at 17 min of the E. coli chromosome (Fig. 10). Since mutations in the loci tolQRAB have been reported to have a phenotype of increased detergent sensitivity and leakiness of periplasmic enzymes, complementation studies with plasmids carrying the wild-type alleles of tolQRAB genes were carried out. The lky mutations in strains K3 and K86 were complemented by plasmids carrying tolB$^+$ and tolA$^+$, respectively (Table 5). These results indicated that the lky mutant K3 was a tolB mutant and the lky allele in K86 was an amber allele of tolA.

tolA mutants are tolerant to all group I colicins including E1, E2, E3, A, and K, while tolB mutants are sensitive to colicin E1 but tolerant to all other group I colicins. Like tolB236, K3 was sensitive to colicin E1, but tolerant to all other group I colicins including E2, E3, A, and K. Like tolA207, K86 was tolerant to all group I colicins. K3 containing a cloned tolB$^+$ gene and K86 containing a cloned tolA$^+$ gene both became sensitive to all group I colicins. These results further confirmed the lky mutations of K3 and K86 as new alleles of tolB and tolA, respectively.

5. Mutation affecting the formation of the murein-bound lipoprotein

Although plasmids carrying tolA$^+$ or tolB$^+$ genes complemented the

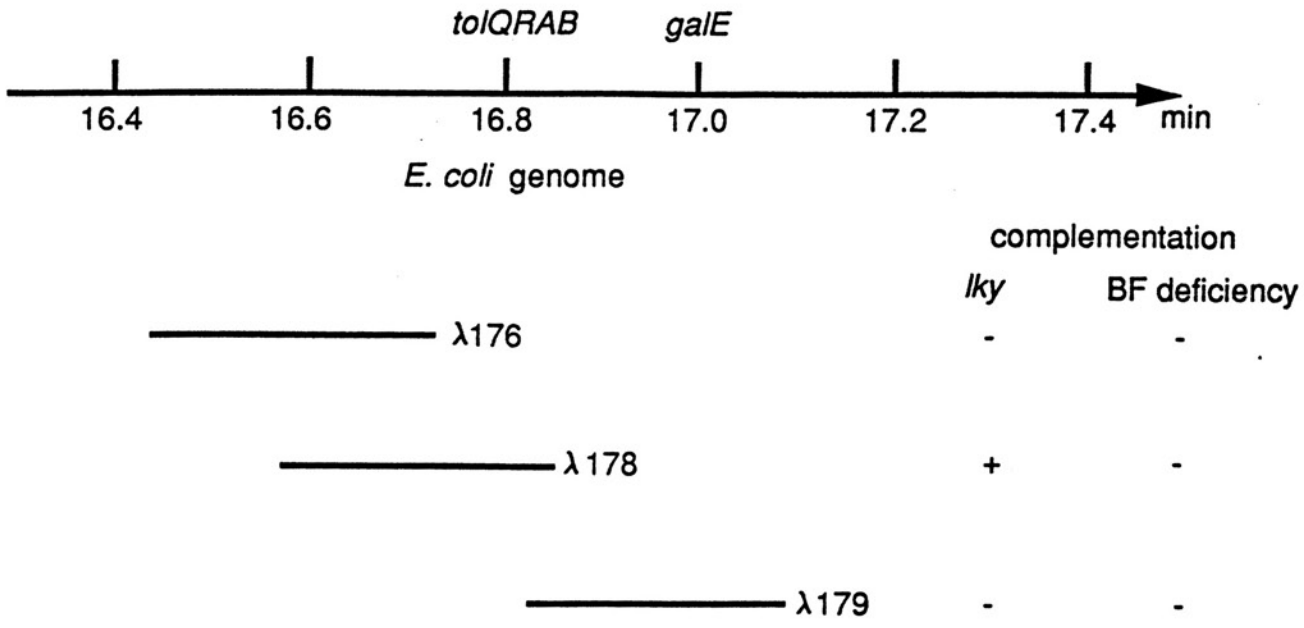


Fig. 10. Complementation of the lky and bound-form lipoprotein deficiency in mutant strains K3 and K86 by Kohara library.

Table 5. Complementation of *tol* and *lky* mutations with cloned *tol*$^+$ genes

Mutant	complementation by plasmid	
	pSKL7f *tolA*$^+$	pNJ1 *tolB*$^+$
JC3411 *tolA*207	+	-
JC3417 *tolB*236	-	+
K3	-	+
K86	+	-
MTA	-	-

lky mutations of K3 and K86 strains, they did not complement the mutation(s) affecting the formation of murein-bound lipoprotein in K3 and K86 strains (Table 6). Furthermore, the bound-form lipoprotein contents of JC3411 (tolA207) or JC3417 (tolB236) were similar to that of the wild-type strain (Table 6). These data suggested that the reduced bound-form lipoprotein in mutants K3 and K86 did not result from the tol mutations. However, the defect in the bound-form lipoprotein formation in mutants K3 and K86 was complemented by the F' factor from strain 5104, 5549, and 4253 but not by the F' factor from strain 4287 (Table 6 and Fig. 9). In addition, the lky mutations of K3 and K86 could be transferred into the wild-type parental strain K37 by P1 transduction (selected for nadA::Tn10), and the transductants also contained reduced amount of bound-form lipoprotein (Table 6). These results suggested that mutant K3 and K86 contained a second mutation at 17 min of E. coli chromosome which resulted in a reduced amount of the bound-form lipoprotein in these mutants.

6. Mapping of the mutation affecting the formation of bound-form lipoprotein in strain MTA.

F⁻ AB1157 was used as a recipient in conjugation to map the lky mutation in MTA (as the Hfr donor). The lky mutation of MTA was found to be linked to the mtl and xyl regions between 80 to 90 min on the E. coli chromosome (data not shown). Results of P1 transduction indicated that the lky mutation in mutant strain MTA was linked to fadA (Table 4). The lky mutation in strain MTA was

Table 6. Murein-bound lipoprotein content in K3, K86 and related strains

strain	murein-bound lipoprotein content (% of wild-type)
K37 (wild-type)	100
K3 (pNJ1 *tolB*$^+$)	48
K86 (pSKL7f *tolA*$^+$)	36
K3 F'4253	100
K3 F'4287	34
K3 F'5104	100
K3 F'5549	93
K86 F'4253	89
K86 F'4287	37
K86 F'5104	100
K86 F'5549	87
K37 *lky* (K3)	37
K37 *lky* (K86)	41
JC3411 *tolA*	100
JC3417 *tolB*	95

distinct from the tol mutations since it was not complemented by plasmid carrying wild-type alleles of tolA or tolB (Table 5), and the MTA strain was sensitive to all group I colicins. However, the lky mutation in mutant strain MTA was also not responsible for the reduced bound-form lipoprotein; with strain 18496 fad::Tn10 as the donor, both the lky and the lky$^+$ transductants of MTA contained similarly low bound-form lipoprotein as the original MTA mutant (Table 7). Likewise, using MTA lky fadA::Tn10 strain as the donor, the lky transductants of the wild-type strain E609 contained normal amounts of bound-form lipoprotein (Table 7). Kohara λ clone 550 containing a 15 kb DNA fragment around 86 min of the E. coli chromosome complemented the lky mutation of MTA mutant but not the mutation affecting the formation of bound-form lipoprotein (Table 7). On the other hand, a gal$^+$ transconjugant obtained by conjugation using MTA as the donor and AB1157 as the recipient exhibited a low bound-form lipoprotein content (Table 7). P1 transductions of MTA using E. coli strains carrying Tn10 insertions between 13.25 and 20 min of the chromosome as donors revealed that the mutation responsible for the low bound-form lipoprotein in mutant MTA was located between 17.75 to 18.75 min of the genetic map (Table 8). In addition, the mutation in MTA affecting the attachment of lipoprotein to the peptidoglycan could be transferred into a wild-type E. coli strain by P1 transduction using MTA mutants containing Tn10 insertions at 17.75 and 18.75 min of the chromosome as the donors (Table 8). Thus the mutation responsible for the reduced amount of bound-form lipoprotein in MTA mutant is

Table 7. Murein-bound lipoprotein content in MTA mutant and related strains

strain	RNase-lky	Bound-form lipoprotein (% of wild-type)
E609 (wild-type)	-	100
MTA	+	24
MTA *fad*::Tn*10* *lky*	+	25
MTA *fad*::Tn*10* *lky*$^+$	-	24
E609 *fad*::Tn*10* *lky*(MTA)	+	95
E609 *fad*::Tn*10* *lky*$^+$	-	94
MTA (λ550)	-	26
AB1157 *gal*$^+$(MTA)	+	27

Table 8. Mapping of the mutation affecting the bound-form lipoprotein in strain MTA by P1 transduction

Donor strain	Recipient strain	Selected marker	Unselected marker	Cotransduction frequency (%)
12021(13.25'::Tn*10*)	MTA	Tc^r	normal BF content	0
12077(15.75'::Tn*10*)	MTA	Tc^r	normal BF content	0
18493(17.75'::Tn*10*)	MTA	Tc^r	normal BF content	60
12034(18.75'::Tn*10*)	MTA	Tc^r	normal BF content	40
18478(20'::Tn*10*)	MTA	Tc^r	normal BF content	0
MTA(17.75'::Tn*10*)	E609	Tc^r	reduced BF content	50
MTA(18.75'::Tn*10*)	E609	Tc^r	reduced BF content	30

The bound-form content was determined by the double-labeling method with ten transductants tested for each P1 transduction.

near the gal region of the E. coli chromosome.

II. Amino-terminal lpp mutations affecting the formation of bound-form lipoprotein.

A. The relationship between modification/processing of prolipoprotein and the formation of bound-form lipoprotein.

1. Lipid-modification and the formation of bound-form lipoprotein.

lpp mutants with alterations in the signal sequence of prolipoprotein used in the present study are shown in Fig. 11. Signal sequence mutants lppL20 and lppV20 were previously shown to contain unmodified prolipoprotein (Pollitt et al., 1986). The prolipoprotein of the mutant lppG21 is not modified by lipid since the Cys21 residue, the attachment site for the glyceryl residue, is changed to Gly (Inouye et al., 1983). Likewise, the OmpF-Lpp hybrid protein lacking the lipoprotein consensus sequence of Leu-Ala-Gly-Cys is not modified with lipid (Choi et al., 1987). Analysis of the free-form lipoproteins from mutants lppL20, V20 and G21 by SDS-PAGE confirmed that these mutants contained unmodified prolipoprotein (Fig. 12). SDS-PAGE analysis of the lysozyme-digested murein sacculi in these mutants indicated that a significant amount of the unmodified prolipoproteins in mutants L20, V20 and G21, and the lipid-deficient OmpF-Lpp hybrid protein were attached to the peptidoglycan as the bound-form lipoprotein (Table 9 and Fig. 12). These results strongly suggest that the lipid modification of prolipoprotein or hybrid protein is not essential for the formation of bound-form lipoprotein.

lpp allele	N-terminal Amino Acid Sequence
Wild-type	M K A T K L V L G A V I L G S T L L A G / C S S N
	1 10 20
lppD14	D
lppE14	E
lppR14	R
lppK14	K
lppL20	L
lppT20	T
lppV20	V
lppG21	G
lppA20I23I24	A I I
lppA20I23K24	A I K

OmpF-Lpp

M M K R N I L A V I V P A L L V A G T A N A / A E S S N

1 10 20

Fig. 11. The NH_2-terminal amino acid sequences of the signal-sequence mutant prolipoproteins and OmpF-Lpp hybrid protein. The amino-terminal amino acid sequence of the wild-type prolipoprotein is shown, and the mutated amino acid residue is given for each of the signal sequence mutants. The slashes indicate the processing site of the wild-type prolipoprotein and that of the precursor form of the OmpF-Lpp hybrid protein.

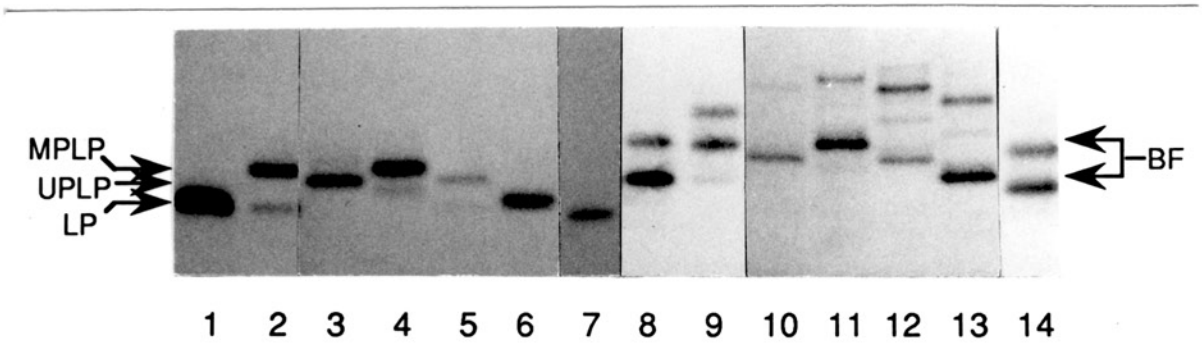


Fig. 12. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from wild-type strain and signal-sequence mutants lppL20, lppT20, lppV20, lppG21, and OmpF-Lpp hybrid protein. Ten μl of the free- and bound-form lipoproteins were loaded in each lane of a Tricine-SDS gel. Lanes 1-7 represent the free-form lipoproteins from the wild-type strain, globomycin-treated wild-type strain, signal-sequence mutant strains L20, T20, V20, G21 and OmpF-Lpp hybrid protein, respectively. Lanes 8-14 represent the bound-form lipoproteins from the wild-type strain, globomycin-treated wild-type strain, mutant strains L20, T20, V20, G21, and the murein-bound OmpF-Lpp hybrid protein, respectively.

Table 9. Murein-bound lipoprotein content in *lpp* signal sequence mutants and a strain containing OmpF-Lpp hybrid protein

Strain	Lpp type*	Bound-from lipoprotein (% of the wild-type)
JA221 pKEN125(wild-type)	LP	100
JA221 pKEN125(globomycin-treated)	MPLP	45
JA221 pL20	UPLP	44
JA221 pT20	MPLP	47
JA221 pV20	UPLP	51
JA221 pG21	UPLP	56
JE5513 pDOC004(*ompF*-*lpp*)	ULP	84

*LP: mature lipoprotein; MPLP: lipid-modified prolipoprotein; UPLP: unmodified prolipoprotein; ULP: unmodified processed OmpF-Lpp hybrid protein.

2. Processing of prolipoprotein and the formation of bound-form lipoprotein.

The prolipoprotein in mutant lppT20 has been shown to be modified with lipid at a reduced rate but not processed (Pollitt et al., 1986). SDS-PAGE analysis of the free-form lipoproteins from globomycin-treated wild-type strain of E. coli and from mutant lppT20 revealed that mutant lppT20 produced two species of free-form lipoproteins: the major species corresponded to the lipid-modified prolipoprotein from globomycin-treated wild-type strain of E. coli (Fig. 12, lane 2), and the minor species migrated to the same position in the gel as that of the unmodified prolipoprotein synthesized in an in vitro transcription/translation system. The lipid-modified prolipoproteins from mutant lppT20 and globomycin-treated wild-type cells were found to be covalently attached to the peptidoglycan (Table 9 and Fig. 12). These results suggest that processing of lipid-modified prolipoprotein is also not a prerequisite for the formation of the bound-form lipoprotein.

B. The effect of charged amino acid residues in the signal sequence of prolipoprotein on the formation of bound-form lipoprotein.

1. Defective formation of bound-form lipoprotein in lppD14 and lppR14 mutants.

In lppD14 mutant, the neutral 14th glycine residue of the signal sequence of prolipoprotein is substituted with a negatively charged Asp (Table 10). The peptidoglycan of mutant lppD14 was shown to contain a greatly reduced amount of mature lipoprotein but

no prolipoprotein (Lin et al., 1980b). Substitution of Gly14 with a positively charged arginine was found to affect the modification and processing of prolipoprotein (Gennity et al., 1990). The formation of bound-form lipoprotein in mutant lppR14 was studied in this thesis. Like LppD14, unmodified prolipoprotein LppR14 is not covalently attached to the peptidoglycan (Table 10 and Fig. 13).

2. Construction and characterization of new mutants lppE14 and lppK14.

To further study the effect of a charged amino acid residue on the formation of bound-form lipoprotein, two signal sequence mutants lppE14 and lppK14 were constructed by oligonucleotide-directed mutagenesis. Replacement of the Gly14 residue with Glu or Lys partially affected the lipid-modification and processing of the mutant prolipoproteins. Seventy percent of the free-form lipoprotein produced by mutant lppK14 was mature lipoprotein (Fig. 13A, lane 4 and Fig 13B, lane 3), while only a small fraction of the lipoprotein in mutant lppE14 was the mature form (Fig. 13A, lane 3 and Fig. 13B, lane 4). The peptidoglycan of the lppE14 and lppK14 mutant cells contained 23% and 74% of mature lipoprotein as that of the wild-type strain but no mutant prolipoprotein (Table 10 and Fig. 13A, lanes 10 and 11). Globomycin-treated lppE14 and lppK14 mutants accumulated both unmodified and lipid-modified prolipoproteins which were not covalently linked to the peptidoglycan (Fig. 14, lanes 8 and 9). These results are consistent with those obtained with the lppD14 and lppR14 mutants,

Table 10. Effects of the amino-terminal *lpp* mutations on the secondary structure, charge of prolipoprotein and the formation of murein-bound lipoprotein

Mutation	Change in charge	Change in secondary structure	Bound-form (% of wild-type)
*lpp*D14	-1		4
*lpp*E14	-1		23
*lpp*K14	+1		74
*lpp*R14	+1		3
*lpp*A20I23I24		decreased β-turn at the processing site	7
*lpp*A20I23K24		decreased β-turn at the processing site	8

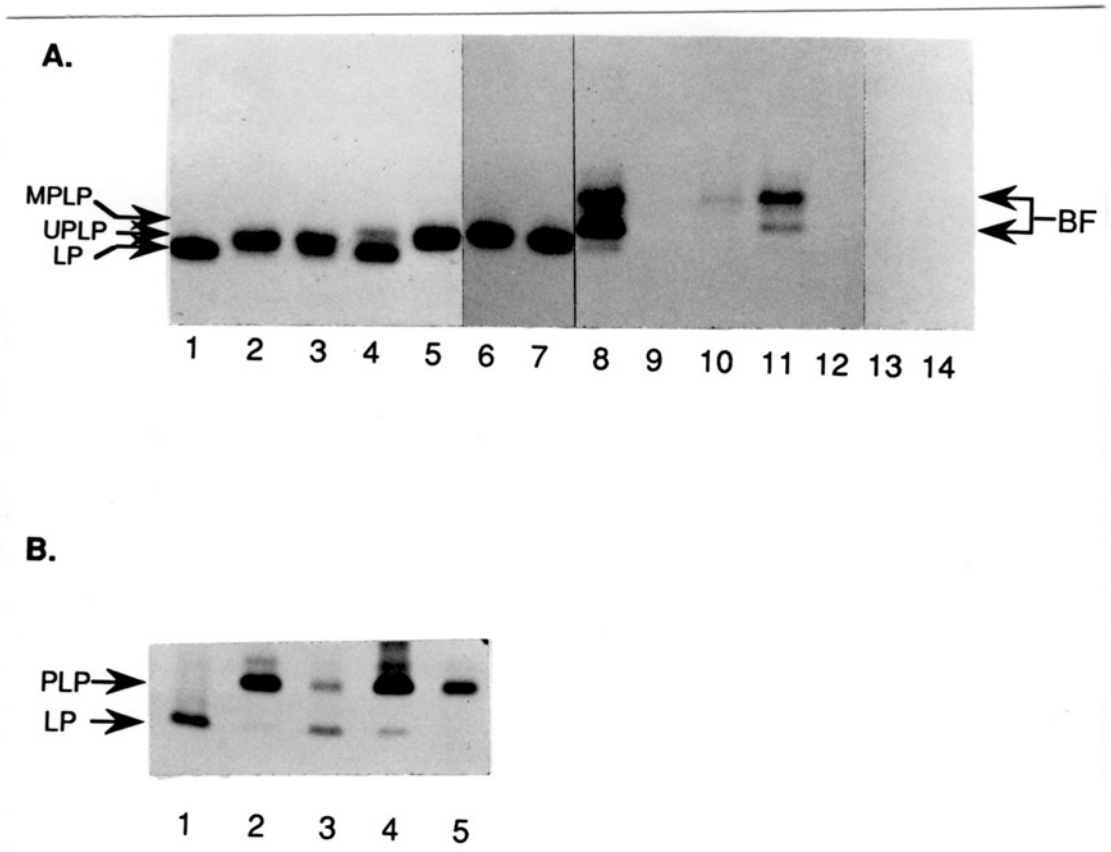


Fig. 13. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from signal sequence mutants lppD14, lppE14, lppK14, lppR14, lppA20I23I24 and lppA20I23K24. In panel A, 6 μl of the free- and bound-form lipoproteins were loaded in each lane of a Tricine-SDS gel. Lanes 1-7, free-form lipoproteins from the wild-type strain, and lpp mutant strains D14, E14, K14, R14, A20I23I24 and A20I23K24, respectively; lanes 8-14, bound-form lipoproteins from the wild-type strain, and lpp mutant strains D14, E14, K14, R14, A20I23I24 and A20I23K24, respectively. In panel B, 6 μl of the free-form lipoproteins were loaded in each lane of a Ito gel. Lanes 1-5, free-form lipoproteins from the wild-type strain, and mutant strains D14, K14, E14 and R14, respectively.

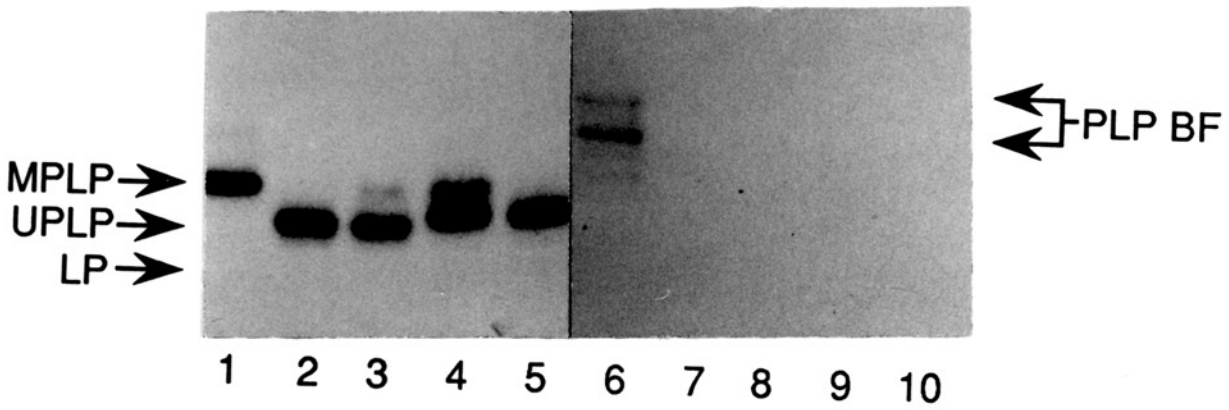


Fig. 14. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from globomycin-treated signal sequence mutants lppD14, lppE14, lppK14 and lppR14. Tricine-SDS gel system was used. Globomycin (100 μg/ml) was added 20 min prior to labeling with [^{35}S]methionine. Six μl of the free- and bound-form lipoprotein samples were loaded in each lane of the gel. Lanes 1-5 represent the free-form lipoproteins from globomycin-treated wild-type strain, mutant strains lppD14, lppE14, lppK14 and lppR14, respectively; lanes 6-10 represent the bound-form lipoproteins from globomycin-treated wild-type strain, mutant strains lppD14, lppE14, lppK14 and lppR14, respectively.

and suggest that a charged amino acid residue at the 14th position of prolipoprotein signal sequence affects the formation of bound-form lipoprotein.

C. The effect of an alteration in the secondary structure of prolipoprotein on the formation of bound-form lipoprotein.

When a silent A20 signal sequence mutation is combined with the I23I24 or I23K24 mutations, the lipid modification and processing of prolipoprotein are completely abolished (Inouye et al., 1986). It has been proposed that a reduction in the predicted β-turn structure at the modification/processing site of the mutant prolipoproteins (Table 10) prevents the lipid modification and processing of the mutant prolipoproteins (Inouye et al., 1986). As shown in Fig. 13A, the unmodified prolipoproteins in mutants A20I23I24 and A20I23K24 were not attached to the peptidoglycan.

III. COOH-terminal lpp mutations and the formation of bound-form lipoprotein.

A. Mutant construction.

The COOH-terminal lysine residue of lipoprotein participates in the covalent linkage between the lipoprotein and the peptidoglycan. Comparison of the amino acid sequences of lipoprotein from five different enterobacteriaceae species including E. coli, P. mirabilis, M. morganii, E. amylovora, and S. marcescens reveals a high degree of sequence homology of the COOH-terminal region of the lipoproteins (Fig. 5); the sequence from A62

to N71 (AARANQRLDN) and Y76 and K78 are conserved, and the 77th amino acid residue is either Lys or Arg.

To ascertain the role of the COOH-terminal amino acid residues in the formation of murein-bound lipoprotein, the amino acid residues at positions 70 (Asp), 75 (Lys), 76 (Tyr), 77 (Arg) and 78 (Lys) were altered by site-specific mutagenesis. The D70 was mutated to E70 which would maintain the negative charge but have an altered secondary structure of the COOH-terminal region of the lipoprotein based on the Chou-Fasman rules (Chou and Fasman, 1978). In contrast, lipoprotein with G70 would have one less negative charge but retain the wild-type secondary structure predicted by Chou-Fasman rules (Chou and Fasman, 1978). To examine the role of the COOH-terminal positively charged amino acid residues in the formation of murein-bound lipoprotein, K75 and R77 were changed to two neutral amino acids, Thr and Leu, respectively. LppR78 mutation was constructed to determine the essentiality of the COOH-terminal Lys residue. In addition, Y76 was altered to Phe or Cys in order to ascertain the role of this conserved amino acid residue in the covalent linkage between lipoprotein and peptidoglycan. The sequences of the oligonucleotides used in the site-specific mutagenesis and the COOH-terminal amino acid sequences of the mutant lipoproteins are shown in Fig. 15.

B. Formation of murein-bound lipoprotein in mutants lppE70, lppG70, lppT75, lppC76, lppF76, lppL77 and lppR78

E. coli strain JA221 carrying the cloned lppC76, L77 and R78

Nucleotide/Amino Acid Sequence

```
Wild-Type      5' CGTCTGGACAACATGGCTACTAAATACCGCAAGTAATAG 3'
                  R  L  D  N  M  A  T  K  Y  R  K  STOP
                  68                          78

lppG70            CGTCTGGGCAACATGGCT
                  R  L  G  N  M  A

lppE70            CGTCTGGAAAACATGGCT
                  R  L  E  N  M  A

lppS70            CGTCTGAGCAACATGGCT
                  R  L  S  N  M  A

lppS75                          GCTACTAGCTACCGCAAG
                                A  T  S  Y  R  K

lppT75                          GCTACTACATACCGCAAG
                                A  T  T  Y  R  K

lppC76                          GCTACTAAATGCCGCAAG
                                A  T  K  C  R  K

lppE76                          GCTACTAAAGAGCGCAAG
                                A  T  K  E  R  K

lppF76                          GCTACTAAATTCCGCAAG
                                A  T  K  F  R  K

lppL77                          GCTACTAAATACCTCAAG
                                A  T  K  Y  L  K

lppD77                          GCTACTAAATACGACAAG
                                A  T  K  Y  D  K

lppR78                                AAATACCGCAGGTAATAG
                                      K  Y  R  R  STOP

lppX76                          GCTACTAAANNNCGCAAG
                                A  T  K  X  R  K
```

Fig. 15. COOH-terminal lpp mutations constructed by oligonucleotide-directed mutagenesis. The altered nucleotides and amino acid residues are underlined. lppX76 are lpp alleles containing mutations at the 76th position of the prolipoprotein obtained by site-specific mutagenesis using mixed oligonucleotide primers containing A, T, C, G at the 76th codon of the prolipoprotein.

mutations exhibited phenotypes of RNase leakiness and increased sensitivity to SDS, while E. coli cells carrying the cloned lpp mutations E70, G70, T75 and F76 were SDS-resistant and RNase-nonleaky (Table 11). In addition, mutants lppC76 exhibited increased resistance to globomycin; the minimum inhibiting concentration (MIC) of globomycin for mutants lppC76 was 80 μg/ml, as compared to 20 μg/ml for the wild-type strain. The bound-form lipoprotein content of lppR78 mutant was negligible as compared to that of the wild-type strain (Table 11 and Fig. 16). This result indicates that the Lys78 residue is essential for the formation of bound-from lipoprotein in E. coli, as expected. In contrast to lppR78, mutants lppE70, G70, T75 and F76 had bound-form lipoprotein contents similar to that of the wild-type strain (Table 11 and Fig. 16). Mutant lppC76 and L77 contained about 15 and 31% of the bound-form lipoprotein as that of the wild-type strain, respectively (Table 11 and Fig. 16). Analysis of the secondary structure of the mutant lipoprotein by Chou and Fasman rules (Chou and Fasman, 1978) revealed that the lppC76 mutant lipoprotein contained an extra β-turn at the COOH-terminal random coil region (Table 12), which might affect the formation of murein-bound lipoprotein.

C. lpp mutations with an extra β-turn at the COOH-terminal region of lipoprotein

To ascertain the effect of a change in the secondary structure of the COOH-terminal region of the lipoprotein on the formation of

Table 11. RNase-leakiness, SDS sensitivity and content of bound-form lipoprotein in lpp mutants with an alteration at the COOH-terminal region of the lipoprotein

Strain	RNase-leakiness	SDS sensitivity	Bound-form as % of wild-type
JA221 p115(wild-type)	-	-	100
JA221 pE70	-	-	100
JA221 pG70	-	-	100
JA221 pT75	-	-	100
JA221 pC76	+	+	15
JA221 pF76	-	-	100
JA221 pL77	+	+	31
JA221 pR78	+	+	4

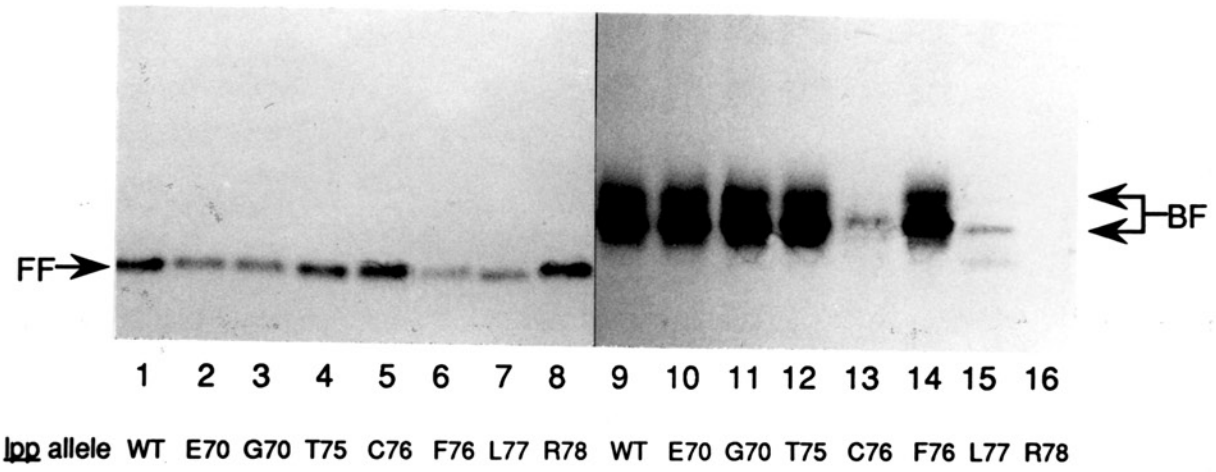


Fig. 16. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from the COOH-terminal mutants. Tricine-SDS gel system was used. Ten μl of the free- and bound-form lipoproteins were loaded in each lane of the gel. Lanes 1-8, free-form lipoproteins; lanes 9-16, bound-form lipoproteins.

Table 12. Effects of the lpp COOH-terminal mutations on the secondary structure and charge of lipoprotein

Mutation	Change of charge	COOH-terminal secondary structure
lppwild-type		random coil
lppE70		shortened random coil
lppG70	+1	random coil
lppS70	+1	β-turn in random coil
lppS75	-1	β-turn in random coil
lppC76		β-turn in random coil
lppG76		β-turn in random coil
lppN76		β-turn in random coil
lppP76		β-turn in random coil
lppS76		β-turn in random coil
lppT75	-1	β-sheet
lppD76	-1	α-helix
lppE76	-1	α-helix
lppH76	+1	α-helix
lppI76		α-helix
lppL76		α-helix
lppD77	-2	β-turn in random coil
lppL77	-1	β-sheet

The predicted changes in the secondary structure of the mutant lipoproteins were based on Chou-Fasman rules (Chou-Fasman, 1978).

the murein-bound form, random mutations at the 76th amino acid residue of the prolipoprotein were isolated by site-specific mutagenesis using mixed oligonucleotide primers containing A, T, C, G at the 76th codon of the lpp gene. Eight mutants were isolated, including lppD76, G76, N76, P76, S76, H76, I76 and L76. lpp mutants G76, N76, P76 and S76 were predicted to contain an extra β-turn at the COOH-terminal random coil region of the lipoprotein, while D76, H76, I76 and L76 lipoproteins were predicted to have a greater propensity for an α-helix at this region (Table 12). E. coli cells carrying the cloned lpp mutations H76, I76 and L76 were not RNase-leaky nor SDS-sensitive, while E. coli cells carrying the cloned lpp mutations G76, N76, P76 and S76 exhibited leakiness of RNase and increased sensitivity to SDS (Table 13). These results suggested that the latter mutants might be defective in the attachment of murein-bound lipoprotein. The content of the bound-form lipoprotein in each mutant was determined. As shown in Table 13 and Fig. 17, mutants lppH76, I76 and L76 contained similar amounts of bound-form lipoprotein as that of the wild-type strain, while the lpp mutants G76, N76, P76 and S76 contained reduced amounts of the murein-bound lipoprotein. These results indicate that an extra β-turn at the COOH-terminal region of the lipoprotein affects the formation of bound-form lipoprotein. However, these mutations which affected both the secondary structure of the COOH-terminal region of the lipoprotein and the formation of the bound-form lipoprotein appeared to be restricted to the last three amino acid residue at the COOH-terminal region. The assembly of murein-

Table 13. RNase-leakiness, SDS sensitivity and content of bound-form lipoprotein in *lpp* mutants containing an altered secondary structure or a charged amino acid residue at the COOH-terminal region of the lipoprotein

Strain	RNase-leakiness	SDS sensitivity	Bound-form as % of wild-type
JA221p115(wild-type)	-	-	100
JA221 pS70	-	-	100
JA221 pS75	-	-	100
JA221 pD76	+	+	23
JA221 pE76	+	+	25
JA221 pG76	+	+	28
JA221 pH76	-	-	91
JA221 pI76	-	-	100
JA221 pL76	-	-	100
JA221 pN76	+	+	32
JA221 pP76	+	+	14
JA221 pS76	+	+	21
JA221 pD77	+	+	21

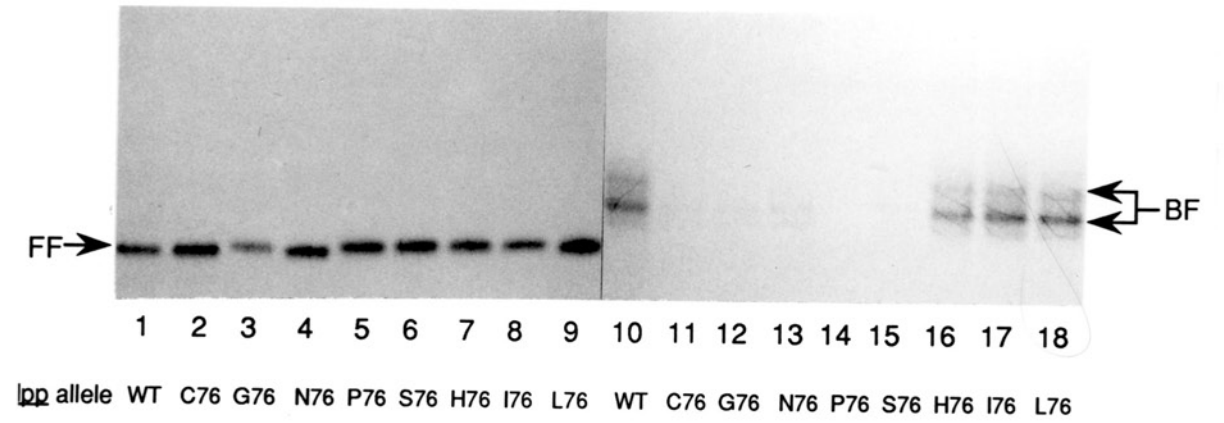


Fig. 17. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from lpp mutants containing an altered 76th amino acid residue at the COOH-terminus. Tricine-SDS gel system was used. Ten μl of the free- and bound-form lipoproteins were loaded in each lane of the gel. Lanes 1-9 represent the free-form lipoproteins. Lanes 10-18 represent the bound-form lipoproteins.

bound lipoprotein were not affected in lpp mutants S70 and S75 (Table 13 and Fig. 18).

D. lpp mutations with a negatively charged amino acid residue at the COOH-terminal region of the lipoprotein.

lpp mutant D76 isolated by random mutagenesis at the 76th codon of the lpp gene was found to be defective in the covalent linkage of lipoprotein to the peptidoglycan (Table 13 and Fig. 19). To ascertain the effect of a negatively charged amino acid residue at the COOH-terminal region of the lipoprotein on the formation of bound-form lipoprotein, two additional lpp mutants, E76 and D77, were constructed by oligonucleotide-directed mutagenesis. Both mutant strains lppE76 and lppD77 contained reduced amount of bound-form lipoprotein (Table 13 and Fig. 19). These results suggest that the introduction of a negatively charged amino acid residue at the positively charged COOH-terminal region of the lipoprotein affects the covalent attachment of free-form lipoprotein to the peptidoglycan.

IV. The formation of bound-form lipoprotein in a lppΔ37-57 deletion mutant.

A. Lipid-modification and processing of the deletion mutant prolipoprotein.

The E. coli pgsA3 allele encoding a defective phosphatidylglycerol phosphate synthase is a lethal mutation for all but certain E. coli strains such as strain SD312. The pgsA3

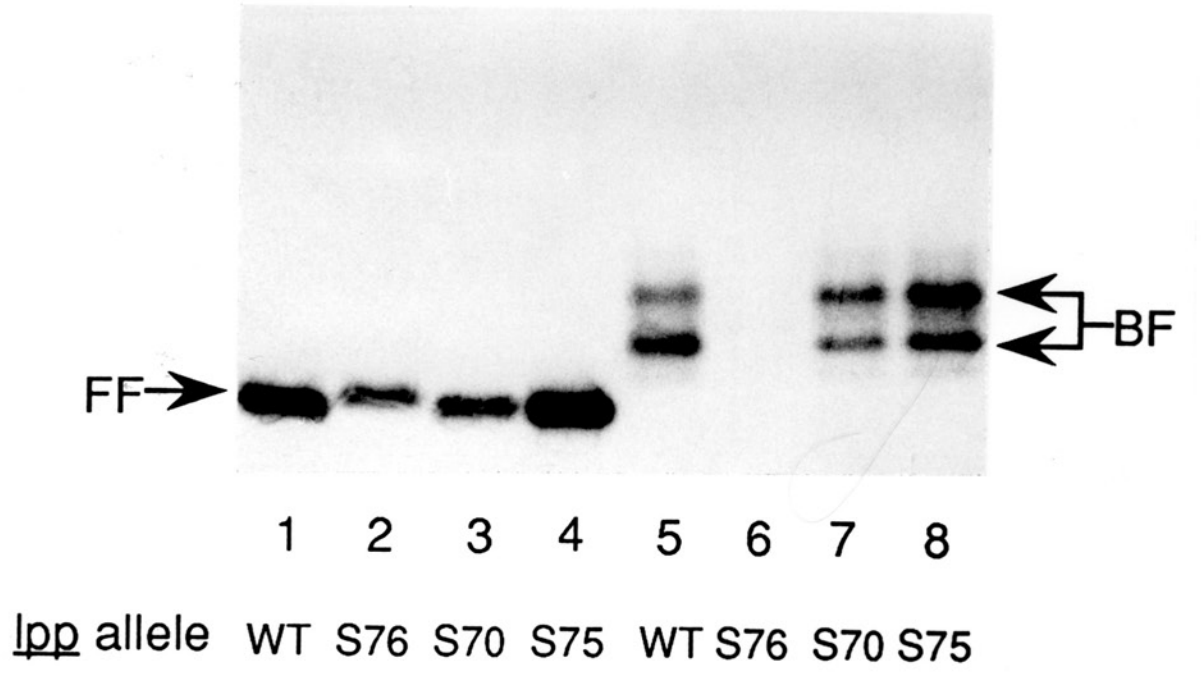


Fig. 18. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from COOH-terminal mutants lppS70 and lppS75. Tricine-SDS gel system was used. Eight μl of the free-and bound-form lipoproteins were loaded in each lane of the gel. Lanes 1-4, free-form lipoproteins; lanes 5-8, bound-form lipoproteins.

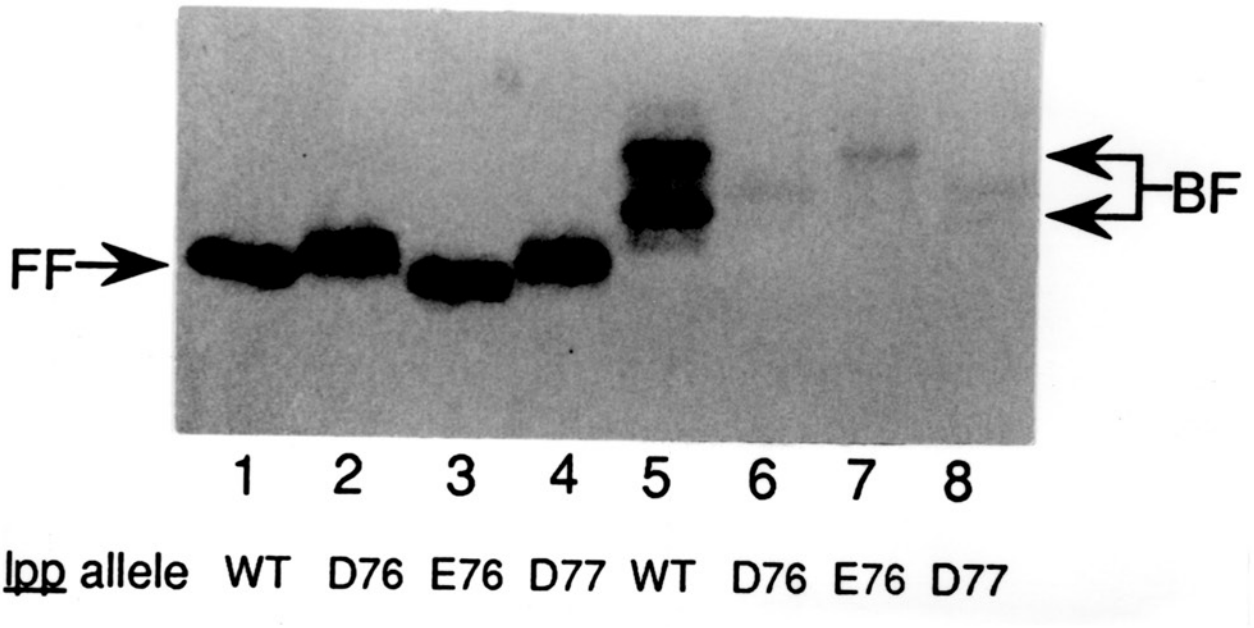


Fig. 19. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from lpp mutants containing a negatively charged amino acid residue at the COOH-terminal region. Tricine-SDS gel system was used. Eight μl of the free- and bound-form lipoproteins were loaded in each lane of the gel. Lanes 1-4, free-form lipoproteins; lanes 5-8, bound-form lipoproteins.

allele could also be introduced into E. coli strains with lpp mutations affecting the synthesis or modification of prolipoprotein (Asai et al., 1989). Ouchterlony tests of the crude extract of strains SD12 and SD312 revealed that SD12 contains a significant amount of lipoprotein while SD312 produces negligible amounts of lipoprotein (Dai and Wu, unpublished data). Direct sequencing of the products of the asymmetric polymerase chain reaction using genomic DNA from E. coli strains SD12 and SD312 pgsA3 as the template showed that the lpp-12 mutation in these two strains consisted a 63 base pair deletion corresponding to the 37th to 57th codons of the wild-type lpp gene (Dai and Wu, unpublished data). The amino acid sequences of the prolipoproteins from the wild-type strain and lppΔ37-57 mutants are shown in Fig. 20. [^{3}H]Palmitate labeling of strain SD12 showed that the shortened prolipoprotein in SD12 strain was modified with lipid (Fig. 21, lanes 2 and 4). Since SD312 strain contained a mutation affecting phosphatidylglycerol phosphate synthesis (Asai et al., 1989), the prolipoprotein of SD312 was not modified with lipid (Fig. 21, lanes 3 and 6). SDS-PAGE analysis of [^{35}S]methionine-labeled lipoproteins from SD12 and SD312 with or without globomycin treatment revealed that the lipid-modified prolipoprotein of SD12 was processed by signal peptidase II (Fig. 22). In contrast, the unmodified prolipoprotein of mutant SD312 was not processed (Fig. 22).

B. Formation of murein-bound lipoprotein in lpp internal deletion mutants.

```
lpp wild-type   MKATKLVLGAVILGSTLLAGCSSNAKIDQLSSDVQTLNA
LPP Δ37-57      MKATKLVLGAVILGSTLLAGCSSNAKIDQLSSDVQT---
                1         10         20         30

                KVDQLSNDVNAMRSDVQAAKDDAARANQRLDNMATKYRK
                ------------------AKDDAARANQRLDNMATKYRK
                40         50         60         70
```

Fig. 20. The amino acid sequences of the prolipoproteins from the wild-type strain and lppΔ37-57 mutant. The dashed line represents the deleted amino acid residues in the prolipoprotein of lppΔ37-57.

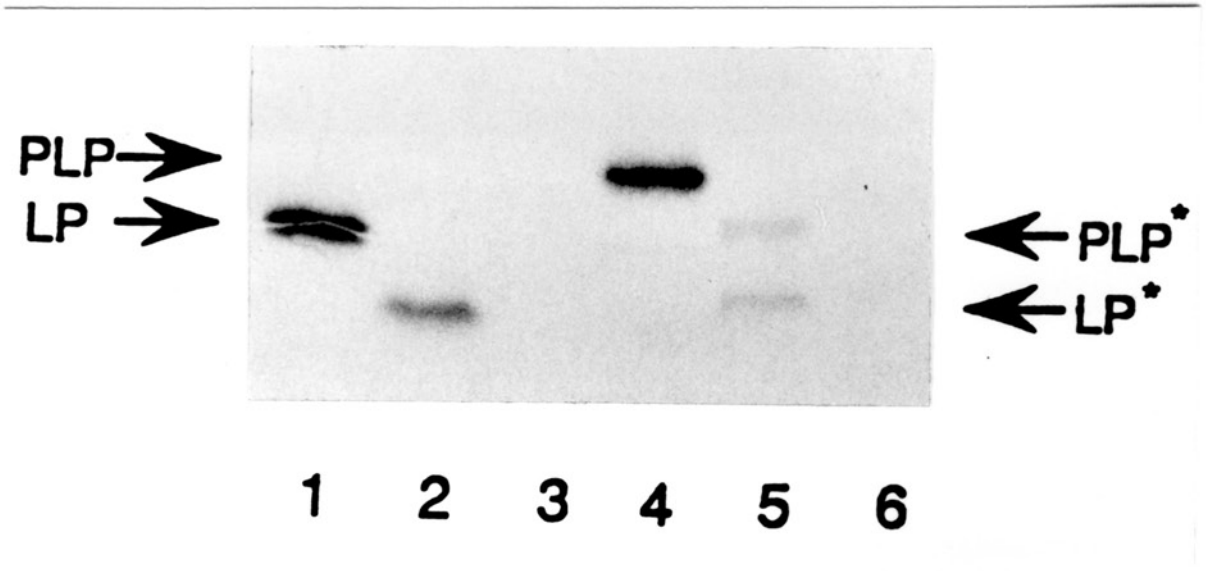


Fig. 21. SDS-polyacrylamide gel electrophoresis of [^{3}H]palmitate-labeled lipoprotein and prolipoprotein from lpp internal deletion mutant strains SD12 and SD312. Five ml cultures of the wild-type strain E613, mutants SD12 and SD312 were labeled with 200 μCi [^{3}H]palmitate at A_{600nm}=0.3 for 1 hr. Globomycin (80 μg/ml) was added 20 min prior to the labeling. Ten μl of the lipoprotein and prolipoprotein samples were loaded in each lane of a Tricine-SDS gel. Lanes 1-3 represent the free-form lipoproteins from E613, SD12, and SD312, respectively, and lanes 4-6 represent the prolipoproteins from globomycin-treated E613, SD12, and SD312, respectively. PLP, modified prolipoprotein; LP, mature lipoprotein; PLP*, modified lppΔ37-57 prolipoprotein; LP*, lppΔ37-57 mature lipoprotein.

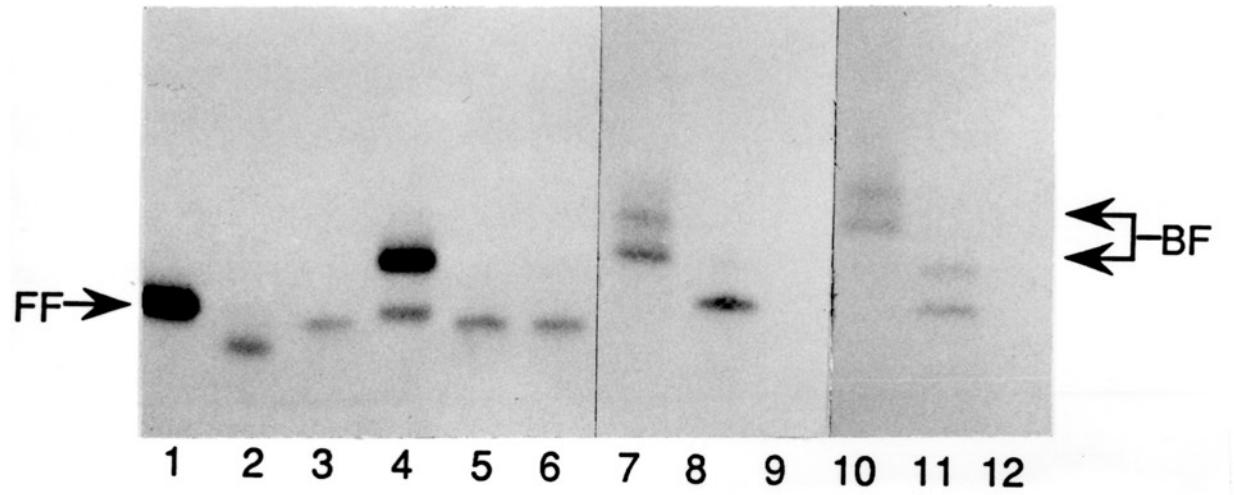


Fig. 22. SDS-polyacrylamide gel electrophoresis of the free- and bound-form lipoproteins from SD12 and SD312 mutant strains with or without globomycin treatment. SDS-Tricine gel system was used. Ten μl of the free- and bound-form lipoproteins were loaded in each lane of the gel. Lanes 1-3, free-form lipoproteins from the wild-type strain E613, and mutant strains SD12 and SD312, respectively; lanes 4-6, prolipoproteins from globomycin-treated E613, SD12 and SD312, respectively; lanes 7-9, bound-form lipoproteins from E613, SD12 and SD312, respectively; lanes 10-12, murein-bound prolipoproteins from globomycin-treated E613, SD12 and SD312, respectively.

The shortened free-form lipoprotein of strain SD12 was found to be covalently attached to the peptidoglycan (Table 14 and Fig. 22, lane 8). The lipid-modified prolipoprotein from globomycin-treated SD12 cells was also covalently attached to the peptidoglycan (Table 14 and Fig. 22, lane 11). These results suggest that the deletion of internal twenty-one amino acid residues of prolipoprotein did not affect the formation of murein-bound lipoprotein. The unmodified prolipoprotein from strain SD312 did not form significant amounts of murein-bound lipoprotein (Table 14 and Fig. 22, lanes 9 and 12). This might result from a defective translocation of prolipoprotein in pgsA3 mutant cells.

Table 14. Murein-bound lipoprotein contents in lpp internal deletion mutants

Strain	Murein-Bound lipoprotein (% of wild-type strain)
E613(wild-type)	100
SD12	82
SD312	8
globomycin-treated 613	47
globomycin-treated SD12	36
globomycin-treated SD312	6

DISCUSSION

I. *lky* mutations in strains K3, K86 and MTA

While Braun's lipoprotein represents one of the most extensively studied proteins in *E. coli*, its precise function remains poorly defined. It has been suggested that the bound-form lipoprotein forms a covalently anchored core of subunits to which the cell wall is built (Braun, 1973; Yamada and Mizushima, 1977). This implies an important, if not essential, role for the bound-form lipoprotein for the maintenance of the structural or functional integrity of the *E. coli* outer membrane. It has also been suggested that the normal invagination of the outer membrane during septation requires the covalent attachment of lipoprotein to the murein layer (Weigand and Rothfield, 1976).

Using transposon and chemical mutagenesis, I have isolated and partially characterized five mutants altered in the assembly of murein-bound lipoprotein. Two of these mutants, E30 and K20, were *lpp* mutants containing Tn*10* or Tn*5* insertion, while the other three mutants, K3, K86 and MTA contained *lky* mutations at the 17 min (*nadA-gal* region) and the 86 min of the *E. coli* chromosome, respectively. K3 and K86 were new alleles of *tolB* and *tolA*, respectively. However, the reduced bound-form lipoprotein in mutants K3 and K86 was not related to the *tolB* and *tolA* mutations. The bound-form lipoprotein contents of other *tolA* or *tolB* mutants were similar to that of the wild-type strain. Plasmids pNJ1 and pSKL7f carrying only *tolA*$^+$ and *tolB*$^+$ complemented the *lky* mutations

in strain K86 and K3, respectively, but did not complement the deficiency in murein-bound lipoprotein in strains K86 and K3. Both the lky mutation and the mutation affecting the formation of bound-form lipoprotein in strains K3 and K86 were complemented by F' 5104, 5549, and 4253 but not by F' 4287. F' 5104, 5549, and 4253 contained overlapping regions of the E. coli chromosome at 17 min (Fig. 9). These data indicate that the reduced bound-form lipoprotein in mutants K3 and K86 is most likely due to a separate mutation located in 17 min region of the E. coli chromosome.

The lky mutation in strain MTA is distinct from the tolQRAB gene cluster, since plasmids carrying the $tolQ^+$, $tolR^+$, (data not shown), $tolA^+$, or $tolB^+$ genes failed to complement the lky mutation of strain MTA, and the MTA strain was sensitive to f1 phage and group I colicins. Strain MTA contained at least two independent mutations. The lky mutation in strain MTA, which mapped at the fadA region of E. coli chromosome, affected the integrity of the cell envelope, but did not affect the formation of the murein-bound lipoprotein. The second mutation affecting the bound-form lipoprotein in MTA was probably also near the gal region (17-18 min) of the E. coli chromosome based on the results of P1 transduction experiments.

Evidence is accumulating which strongly suggest that the gene(s) involved in the formation of murein-bound lipoprotein is located in 17 min region of the E. coli chromosome. A SDS^r revertant of lppG76 mutant has been isolated, and the revertant contains nearly normal amounts of murein-bound lipoprotein. The

reversion was found to be unlinked to the lpp gene on the plasmid, and mapped at 12-17 min region of E. coli chromosome by conjugation (Lu and Wu, unpublished data). The failure of Kohara λ clone 176, 178 and 179 containing overlapping sequence between 16.4-17.1 min of E. coli chromosome to complement the mutation affecting the bound-form lipoprotein in K3 and K86 suggests that the latter mutation is located beyond 17.1 min of the E. coli chromosome. The mutation affecting the formation of the bound-form lipoprotein in strain MTA mapped between 17.75 to 18.75 min by P1 transduction (Table 8). Kohara clones containing inserts in 17-18 min region of the E. coli chromosome would be useful for further fine mapping and cloning of the gene(s) involved in the formation of murein-bound lipoprotein.

The amount of murein-bound lipoprotein in all three lky mutants is not very low; the bound-form lipoprotein of mutant MTA was about one fourth that of the wild-type parental strain. This apparent leakiness in the bound-form lipoprotein formation might be due to the presence of multiple L,D-transpeptidases (lipoprotein-peptidoglycan ligases) in E. coli, similar to the existence of multiple transglycosylase-transpeptidases for the biosynthesis of peptidoglycan in E. coli. Mutation in one of the genes encoding transpeptidases would result in a partial defect in the formation of bound-form lipoprotein in the mutant strain. Alternatively, the mutation affecting the bound-form lipoprotein in strains K3, K86 and MTA alters the synthesis or activity of a single lipoprotein-peptidoglycan ligase, but only partially. Resolution of this

question awaits the biochemical and genetic characterization of the mutations in K3, K86 and MTA affecting the formation of murein-bound lipoprotein.

II. lpp signal sequence mutations and the formation of bound-form lipoprotein

The covalent attachment of lipoprotein to the peptidoglycan is the last step of the biosynthesis of murein lipoprotein in E. coli. It has been suggested that the assembly of lipoprotein into the murein sacculus requires a prior modification and processing of lipoprotein (Lin et al., 1980b). In the present study, I have examined the relationship between the modification/processing of prolipoprotein and the formation of bound-form lipoprotein. The results summarized in Table 15 indicate that the unmodified prolipoprotein (in mutants lppL20, V20, and G21), the lipid modified prolipoprotein (in mutant lppT20 and globomycin-treated wild-type cells), and the lipid-deficient OmpF-Lpp hybrid protein are all assembled into the murein sacculi, albeit less efficiently than the mature lipoprotein. In addition, apolipoprotein from a S. typhimurium mutant defective in the N-acylation of apolipoprotein was also found to form covalent linkage with the peptidoglycan (Gupta and Wu, unpublished data). We can conclude, therefore, that neither the lipid modification nor the processing of prolipoprotein is essential for the formation of bound-form lipoprotein. As shown in Table 15, the mature free-form lipoprotein is most efficiently assembled into the murein sacculi. Other forms of lipoprotein

including lipid-modified prolipoprotein, unmodified prolipoprotein, and lipid-deficient OmpF-Lpp hybrid protein are not as good substrates as the mature lipoprotein for the lipoprotein-peptidoglycan ligase (Table 15).

Mutant Lpp containing a charged amino acid residue Asp at the 14th position of the signal sequence was found to be defective in the formation of the bound-form lipoprotein (Lin et al., 1980b). Similar results were obtained in the present study with lppR14 mutant (Table 10 and Fig. 13). This conclusion is further supported by the observation that unmodified or lipid-modified prolipoproteins in new mutants lppK14 and lppE14 were not linked to the peptidoglycan (Fig. 14). Since neither the lipid modification of prolipoprotein nor the removal of the signal sequence per se is required for the covalent attachment of prolipoprotein to the peptidoglycan, the defective assembly of mutant prolipoproteins in lppD14, lppE14, lppK14, lppR14 must be attributed to the presence of a charged residue in the signal sequence of the mutant prolipoproteins.

Mutant prolipoproteins in lppA20I23I24 and lppA20I23K24 were also not assembled into the murein sacculus (Table 10 and Fig. 13A). It has been suggested that an altered secondary structure in the vicinity of modification/processing site in lppA20I23I24 and lppA20I23K24 prolipoproteins affects the lipid-modification of these two mutant prolipoproteins (Inouye et al., 1986). Our results suggest that the β-turn secondary structure at the modification/processing site is also important for the formation

Table 15. Summary of the experimental data on the modification/processing of prolipoprotein and the formation of bound-form lipoprotein in *lpp* mutants

Strain (*lpp* allele)	Modification	Processing	BF %	Lipoprotein form*
Wild-type	+	+	100	LP
Globomycin-treated wild-type	+	-	45	MPLP
L20	-	-	44	UPLP
T20	+	-	47	MPLP
V20	-	-	51	UPLP
G21	-	-	56	UPLP
OmpF-Lpp	-	+	84	ULP
D14	-	-	4	LP
R14	-	-	3	LP
E14	+/-	+/-	23	LP
K14	+/-	+/-	74	LP
A20I23I24	-	-	7	LP
A20I23K24	-	-	8	LP

*: LP, lipoprotein; UPLP, unmodified prolipoprotein; MPLP, lipid-modified prolipoprotein; ULP, unmodified OmpF-Lpp hybrid protein.

of bound-form lipoprotein.

It is not clear how the introduction of a charged amino acid at the 14th position of the signal sequence of the prolipoprotein or an alteration in the secondary structure of the prolipoprotein in the vicinity of the modification/processing site affects the formation of bound-form lipoprotein. They may affect the interaction between the mutant prolipoprotein and the putative lipoprotein-peptidoglycan ligase. Alternatively, a trimeric form of lipoprotein in the outer membrane or periplasm (Choi et al., 1987) may be the actual substrate in the formation of bound-form lipoprotein, resulting in a free- to bound-form ratio of 2:1. Mutant prolipoprotein with an extra charge or a more rigid secondary structure in the signal sequence may be defective in the formation of such a trimer. Consequently, they are deficient in the formation of bound-form lipoprotein.

The amounts of the mature lipoproteins attached to the murein sacculus were different among the four signal sequence mutants, lppD14, lppE14, lppK14 and lppR14; they contained about 4%, 23%, 74% and 3% of mature bound-form lipoprotein as that of the wild-type strain, respectively (Table 10). These differences in the bound-form lipoprotein contents reflect corresponding differences in the modification and processing of these mutant prolipoproteins, and prolipoproteins containing a charged 14th amino acid residue in *these mutants do not form covalent linkage with the peptidoglycan.* The majority of the free-form lipoprotein produced by mutant K14 was mature lipoprotein, while only a small part of the free-form

lipoprotein in mutant E14 was mature lipoprotein. Mutants D14 and R14 contained insignificant amounts of mature free-form lipoprotein (Fig. 13). The reason for such differences in the modification and processing of these four mutant prolipoproteins is not clear. It may be due to the differences in the pK_a values of these charged amino acids, with the pK_a for the side chains of K, R, D, and E being 10.5, 12.5, 3.9 and 4.3, respectively. The charge differences between Arg and Lys and between Asp and Glu should be insignificant at physiological pH in an aqueous environment. However, the physiological pH in the microenvironment of the prolipoprotein modification and processing enzymes is not known. Functional differences between Arg and Lys have been found in other exported proteins. An Arg residue in the amino terminal region of a β-lactamase-chicken muscle triosephosphate isomerase hybrid protein prevents the secretion of this hybrid protein in E. coli cells, while a Lys residue does not (Summers et al, 1989). Similarly, substitution of Pro with Arg at the amino terminus of the mature alkaline phosphatase (PhoA) of E. coli severely affects the export of PhoA, while substitution of Pro with Lys results in a less severe defect in the export of PhoA (Li et al., 1988).

Alternatively, the differences in the maturation of lipoprotein among these four lpp mutants may be related to the secondary structure of the signal sequences of these mutant prolipoproteins. Previous studies has suggested that a unique secondary structure of a coil region (residues 14, 15) followed by a β-sheet structure (residues 16-18) immediately preceding the β-

turn structure at the modification and processing sites (residues 19-24) of prolipoprotein may be important for the recognition of prolipoprotein by the glyceryl transferase (Giam et al., 1984a). Prediction of the secondary structures of lppD14, E14, K14 and R14 mutant prolipoproteins by the Chou-Fasman empirical method (Chou and Fasman, 1978) indicates that Lpp containing E14 or K14 have a greater probability of forming the coil structure at the 14-15th position of prolipoprotein than those containing D14 or R14 (data not shown). Thus prolipoproteins LppE14 and LppK14 are less defective in lipid-modification and processing than LppD14 and R14.

III. COOH-terminal lpp mutations and the formation of bound-form lipoprotein

Alterations of the COOH-terminal region of the lipoprotein by site-specific mutagenesis indicated that the last three amino acid residues (Tyr76, Arg77 and Lys78) at the COOH-terminus of lipoprotein are important for the formation of the bound-form lipoprotein. Mutations at amino acid residue 70 and 75 of the prolipoprotein (E70, G70, S70, S75 and T75) did not impair the covalent attachment of the lipoprotein to the peptidoglycan, even though these mutational alterations also affected the secondary structure or charge of the lipoprotein. These data suggest that within the carboxy-terminal region of lipoprotein, the Tyr76-Arg77-Lys78 residues play a critical role in the formation of the covalent linkage between the lipoprotein and the peptidoglycan by the putative lipoprotein-peptidoglycan ligase.

Previous studies have suggested that the deletion of the COOH-terminal lysine residue of lipoprotein might weaken the electrostatic interaction between the +2 charge domain of the lipoprotein COOH-terminus (Arg-Lys) with the -2 charged tetrapeptide of peptidoglycan, and result in the loss of both the covalent and noncovalent interactions of the mutant lipoprotein with the peptidoglycan (Choi et al., 1987). The lppR78 mutation, which maintained the +2 charge of the COOH terminus of lipoprotein, was designed to ascertain whether or not the 75th lysine residue could substitute for the Lys78 in the formation of the covalent linkage between the lipoprotein and the peptidoglycan in lppR78 mutant. The fact that the formation of bound-form lipoprotein is totally defective in lppR78 indicates that the COOH-terminal lysine residue is essential for the formation of murein-bound lipoprotein. In the wild-type E. coli background, the Lys75 of the prolipoprotein cannot substitute for the Lys78 in the formation of bound-form lipoprotein.

Among the COOH-terminal lpp mutants, lppC76, lppG76, lppN76, lppP76, and lppS76 contained significantly reduced murein-bound lipoprotein. According to the predicted secondary structure by the Chou-Fasman rules (Chou and Fasman, 1978), these five mutant lipoproteins contained an extra β-turn at the COOH-terminal random coil (Table 12). Either an uninterrupted COOH-terminal random coil is essential for the formation of bound-form lipoprotein, or the extra β-turn at the COOH-terminal region interferes with the attachment of the lipoprotein to the peptidoglycan. Mutant

lipoproteins H76, I76 and L76 which are predicted to have an α-helix rather than a random coil secondary structure at the COOH-terminal region were not affected in the formation of murein-bound lipoprotein. These results do not support that the uninterrupted COOH-terminal random coil is essential for the formation of bound-form lipoprotein but suggest that an extra β-turn at the COOH-terminal region (76-78th residues) interferes with the formation of murein-bound lipoprotein.

Mutant lipoproteins LppS70 and LppS75 which also contained an extra β-turn at the COOH-terminal region were attached to the peptidoglycan. The extra β-turn at the 70th position of prolipoprotein may not be close enough to the COOH-terminal Lys residue to affect the attachment of mutant lipoprotein to the peptidoglycan. On the other hand, secondary structure analysis using the Chou-Fasman rules has revealed that the probability of an extra β-turn in the COOH-terminal region of mutant lipoprotein LppS75 is less than that in the COOH-terminal region of LppS76. Furthermore, LppS75 has a greater tendency to form a β-sheet structure at the COOH-terminal region.

The lipoproteins from five different enterobacterial species including E. coli, P. mirabilis, M. morganii, E. amylovora, and S. marcescens, and from P. aeruginosa all contain a positively charged COOH-terminus, either arginyl-lysine or lysinyl-lysine (Fig. 5.; Yu, 1987; Duchêne et al., 1989). This implies an important role of these positively charged COOH-termini in the covalent attachment of the lipoprotein to the peptidoglycan. Introduction of a negatively

charged amino acid residue at the 76th (D76 and E76) or 77th (D77) position of prolipoprotein resulted in a reduced attachment of free-form lipoprotein to the peptidoglycan. Reduction of the positive charge of the COOH-terminal region by the replacement of R77 with a neutral Leu also affected the formation of bound-form lipoprotein. These results suggest that the positively charged COOH-terminal region participates in an electrostatic interaction with the tetrapeptide of the peptidoglycan, and consequently is important for the formation of murein-bound lipoprotein.

IV. Internal lpp deletion and the formation of bound-form lipoprotein

It is not surprising that the deletion of twenty-one amino acid residues internal to lipoprotein did not affect the formation of murein-bound lipoprotein. Analysis of the amino acid sequence of prolipoproteins from E. coli and four other enterobacteriaceae species reveals that the signal sequence of prolipoprotein, the amino terminal sequence (C21-L30) and the COOH-terminal sequence (A62-K78) of the prolipoprotein are highly conserved, with a sequence homology of 65%, 70% and 71%, respectively. In contrast, the internal sequences of the prolipoproteins (from S31 to D61) has a low (32%) sequence homology (Fig. 5). The results described in this thesis indicate that this less conserved internal sequence of the prolipoprotein is not essential for the formation of murein-bound lipoprotein. The internal sequence of the prolipoprotein is also not essential for the lipid modification and processing of the

prolipoprotein (Fig. 21 and 22). This is consistent with the observation that a Lpp-Bla hybrid prolipoprotein containing the signal sequence plus nine amino-terminal residues of the mature lipoprotein followed by the β-lactamase is lipid modified and processed like the lipoprotein (Ghrayeb and Inouye, 1984).

The pgsA3 mutation in strain SD312 affects the lipid-modification of prolipoprotein due to a defective synthesis of the phosphatidylglycerol phosphate. The defect in the attachment of the unmodified prolipoprotein to the peptidoglycan in strain SD312 is probably not due to the absence of lipid-modification of the mutant prolipoprotein or the internal deletion in the mutant prolipoprotein, since the lipid-modification is not a prerequisite for the formation of murein-bound lipoprotein, and the deletion of internal amino acid residues does not affect the attachment of the mutant lipoprotein to the peptidoglycan in strain SD12. More likely, this defect in the bound-form formation results from a defective translocation of the prolipoprotein across the cytoplasmic membrane of mutant SD312. It has been reported that acidic phospholipids are required for the translocation of precursor proteins in E. coli, both in vivo and in vitro (de Vrije et al., 1988). Translocation of precursor proteins across the cytoplasmic membrane in E. coli requires not only the secretory machinery comprising of SecA, SecY, SecE, SecD and SecF proteins but also acidic phospholipids. Translocation of prolipoprotein is defective in SD312 pgsA3; consequently, the unmodified prolipoprotein in SD312 pgsA3 is not attached to the peptidoglycan

in a reaction presumably occurring in the periplasm.

V. Summary and future studies

Three E. coli mutants (K3, K86 and MTA) with a reduced formation of murein-bound lipoprotein have been identified and partially characterized in this study. These mutations have been mapped to 17-18 min region of the E. coli chromosome. Fine mapping of these allele(s) and cloning of the wild-type allele(s) of these gene(s) will be a logical extension of this study.

The structural requirement of the lipoprotein in the formation of murein-bound form lipoprotein in E. coli has been systematically investigated in this thesis. I have shown that neither the modification nor the processing of prolipoprotein is a prior requirement for the formation of bound-form lipoprotein. On the other hand, the presence of a charged amino acid residue at the 14th position of the signal sequence of the prolipoprotein inhibits the formation of bound-form lipoprotein. In addition, an alteration in the secondary structure of the prolipoprotein in the vicinity of the modification/processing site inhibits the formation of bound-form lipoprotein.

The highly conserved carboxy-terminal region of the lipoprotein has been systematically altered by site-directed mutagenesis in order to define the structural features essential to the formation of bound-form lipoprotein. The results indicate that the conserved COOH-terminal amino acid residues (Tyr76-Arg77-Lys78) are important for the formation of murein-bound lipoprotein, and

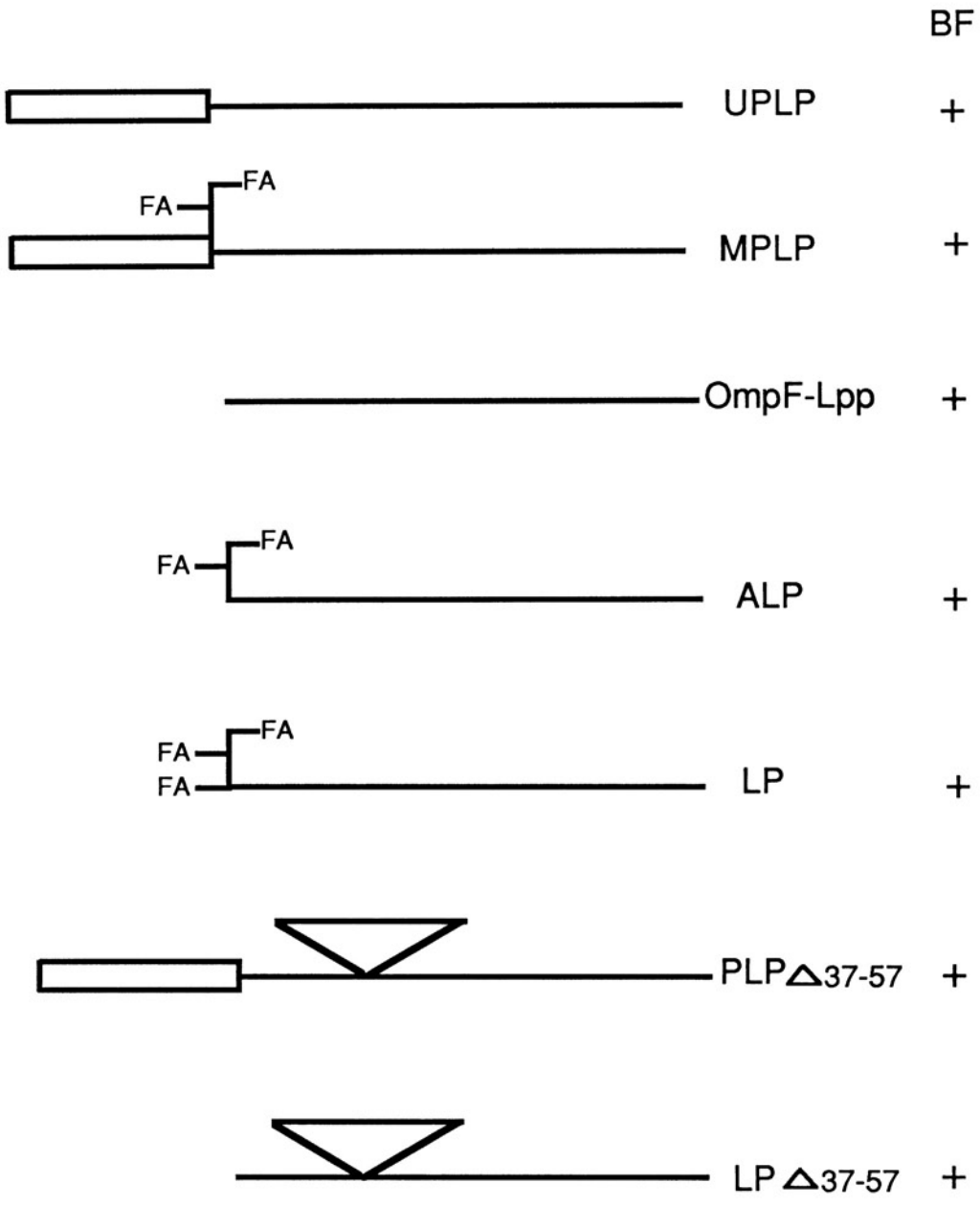
the presence of a β-turn structure in this region results in a reduced formation of the bound-form lipoprotein. In contrast, amino acid residues internal to the prolipoprotein (Leu37-Ala57) are not essential for the attachment of lipoprotein to the peptidoglycan.

The relationship between the structure of (pro)lipoprotein and the formation of murein-bound (pro)lipoprotein is schematically summarized in Fig. 23.

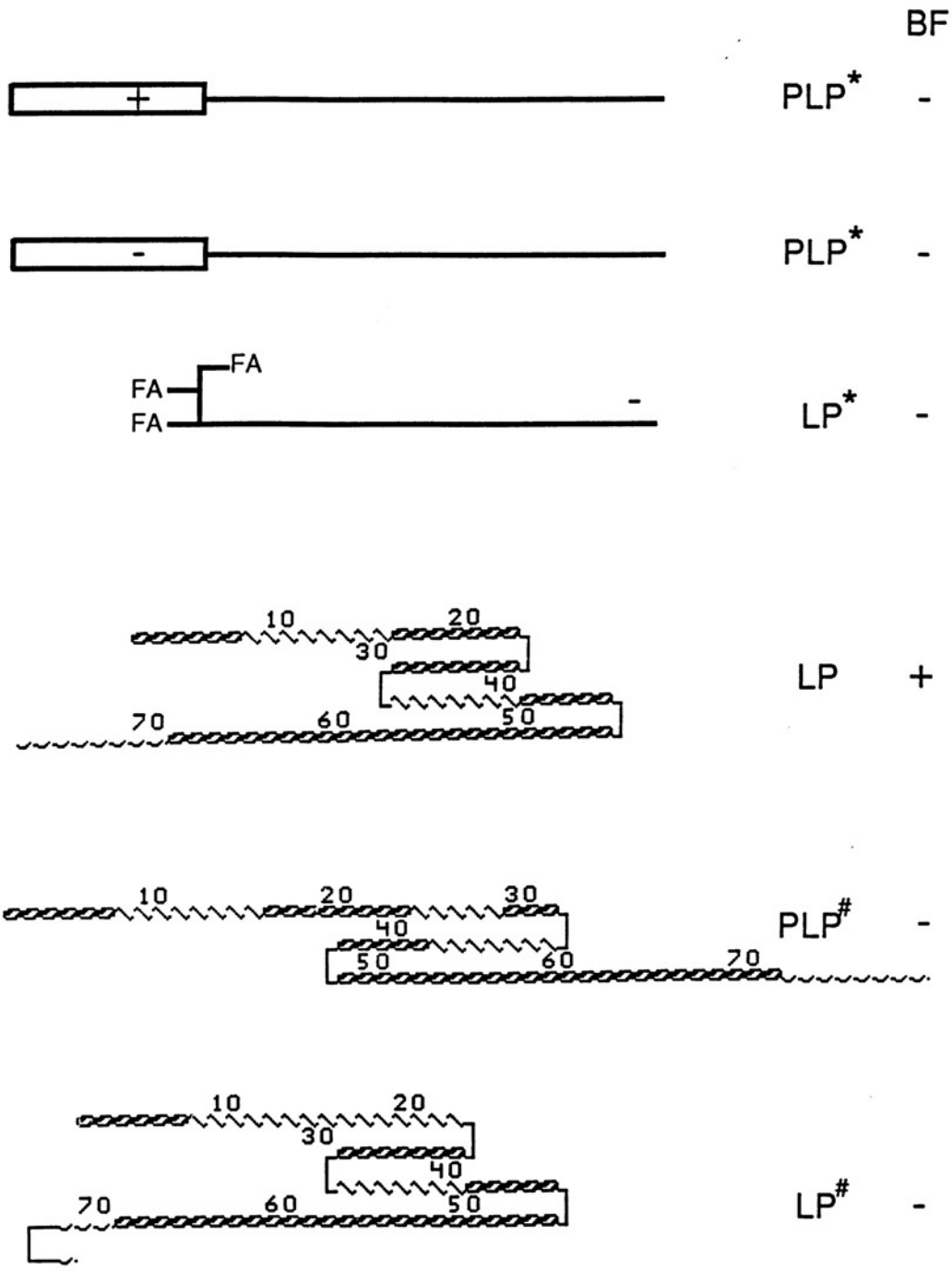
The results obtained in the present study provide the rational for future studies. Isolation and characterization of unlinked suppressors of lppC76, lppG76 and lppS76 would facilitate the identification of the gene(s) of the lipoprotein-peptidoglycan ligase. The suppressor mutations can be mapped by conjugation and P1 transduction, and cloned. Fusion proteins between alkaline phosphatase (PhoA) and varying lengths of the COOH-terminal sequence of the lipoprotein might be a fruitful approach to define the minimum length of the COOH-terminal sequence in lipoprotein for its covalent attachment to the peptidoglycan. PhoA is normally exported to the periplasm. If a phoA-lpp fusion protein consisting of PhoA and COOH-terminal sequence of Lpp is covalently attached to the peptidoglycan, one might detect a difference in the appearance of blue dye in colonies on an X-P plate, especially under conditions which facilitate the leakage and diffusion of periplasmic enzymes (e.g. EDTA treatment). The presence of PhoA in the murein sacculus can also be determined directly by Western blot analysis of lysozyme-digested murein sacculus with anti-PhoA antibodies.

Fig. 23. The relationship between the structure of (pro)lipoprotein and the formation of murein-bound (pro)lipoprotein. Panel A. Lipid-modification and processing of prolipoprotein are not essential for the formation of bound-form lipoprotein. In addition, deletion of internal amino acid sequence does not affect the formation of murein-bound form. Panel B. The effects of charge or alterations in secondary structure of (pro)lipoprotein on the formation of bound-form lipoprotein. UPLP, unmodified prolipoprotein; MPLP, modified prolipoprotein; OmpF-Lpp, unmodified but processed OmpF-Lpp hybrid protein; ALP, apolipoprotein; LP, mature lipoprotein; PLPΔ37-57, prolipoprotein of lppΔ37-57 deletion mutant; LppΔ37-57, mature lipoprotein of lppΔ37-57 deletion mutant; PLP*, mutant prolipoprotein with a charged 14th amino acid residue; LP*, mutant lipoprotein with increased negative charge at the carboxy-terminal region. PLP#, mutant prolipoprotein with an altered secondary structure at the modification/processing site; LP#, mutant lipoprotein with an extra β-turn at the carboxy-terminal region.

A.



B.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